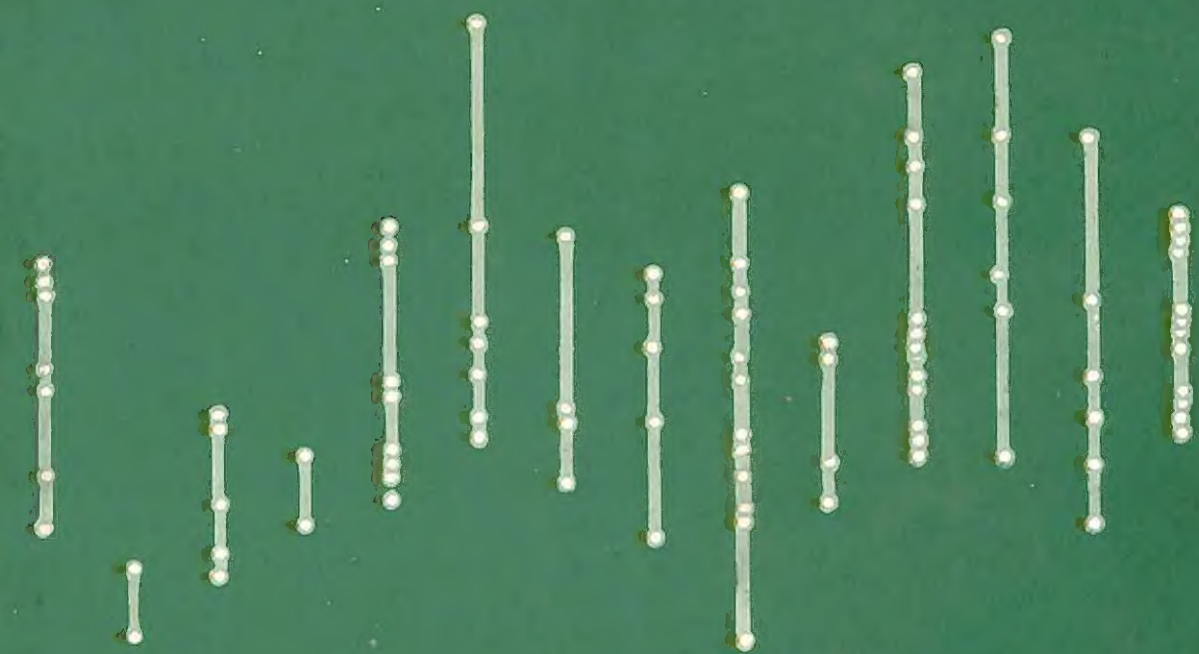
advances in cell and chromosome research



Editors
ARUN KUMAR SHARMA
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Developments in the field of chromosome research are too rapid to enable a reader to keep abreast with progress in all its facets. This series attempts to present the advances in different areas at a given period of time. *Advances in Chromosome and Cell Genetics*, published in 1985, dealt with the progress on split genes, functional differentiation of chromosome sequences, evolution in higher groups, environmental mutagenesis and problems of differentiation, among others.

This book covers advances in other areas of chromosome study as far as practicable. The clarification of organizational elements of chromosomes has led to a deep understanding of chromosome skeleton. The genome organization in the plant system in which "so-called" redundant sequences form inherent components still provides challenging problems. The extent to which environment affects the mutant genes which themselves yield variability is of special interest. Chromosome study in plants in relation to antiquity and geological events is extremely fascinating. In the area of human genetics and genetic toxicology, the clastogenicity of the metals—their synergistic and antagonistic properties, drug toxicity and occupational mutagenicity and carcinogenicity have led to a deeper understanding of environmental problems. The study on xenobiosis has helped in correlating metabolism and carcinogenesis. The continued addition of test systems and DNA adducts in genotoxicity assay are yielding valuable data. Research on skin genetics is an important facet of medical genetics. An attempt has been made to present the advances in all these areas of chromosome research, which are of special value in biology, medicine and agriculture.

ADVANCES IN CELL AND CHROMOSOME RESEARCH

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Preface

Since the publication of *Advances in Chromosome and Cell Genetics* in 1985, developments in the field of chromosome and cell genetics have been remarkable. The advances have affected all aspects of research, both fundamental and applied. Leaving aside the progress in the intervening period, several areas of chromosome research could not be covered in the earlier book.

Concomitant with the advances in our understanding of the finer details of chromosome structure and its genetic elements, innumerable data have been generated on the response of chromosomes and their structural and functional components to environmental influences, both particulare and non-particulate. They may influence mutation, affect the mutator genes or may result in disrupted metabolism, often leading to neoplasia. The techniques of monitoring their effects, including the occupational hazards, through various approaches, involving different test systems and cellular and molecular phenomena such as formation of DNA adducts, have reached a very high level of precision. Discussion on the state-of-the-art on these issues are called for and hence the publication of the present volume is needed.

One of the papers in this volume is by the Late Professor R.O. Whyte, on Botanical Consequences of Tectonics and Orogeny. On the basis of his vast experience, he dealt with the use of chromosome data on evolutionary botany. His sudden death before the article could be finalized prompted his wife Mrs. Pauline Whyte to complete the unfinished task of her husband. This job, she did with meticulous care. Her untimely death as well, prevented her from seeing the article in print. It is a privilege to dedicate this volume to Late Professor R.O. Whyte and Mrs. Pauline Whyte, whose love and affection we cherish.

We, are thankful to Mr. S.K. Sur for helping us in the preparation and typing of the author index.

July, 1989

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1

The Influence of Environmental Factors on the Action of Mutant Genes

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1. Introduction

It is well known that distinct environmental factors can strongly influence the vitality and fertility of mutants. Their behaviour in the normal environment, i.e., under conditions which are more or less optimal for the mother varieties, allows therefore no reliable conclusions on their selection value. Many mutants show a certain degree of elasticity enabling them to adapt themselves to specific ecological conditions. Some of them may be able to survive in distinct ecological niches which are unsuited for their initial lines or for other genotypes of the species. Such a flexibility becomes only discernible if the mutants are comparatively grown under different environmental conditions.

Investigations of this kind have been carried out already some decades ago in plant as well as in animal species with regard to the influence of the temperature. The *yw* mutant of *Drosophila melanogaster*, for instance, was found to have a certain resistance to abnormally high temperatures, which lead to a selection advantage under extreme conditions [77]. The relative vitality of the *Venae abnormes* (*VA*) and *miniature* (*m*) mutants of *Drosophila* decreases, that of mutant *bobbed* (*bb*) increases with increasing temperature [87]. A particularly interesting dependence on the temperature was found for the *short-wing* (*sw*) mutant. The *sw* gene is pleiotropic causing the formation of narrow wings and small eyes and reducing the fertility of the flies at temperatures around 23°C. At temperature conditions around 31°C, gene *sw* acts as a lethal factor. Decreasing temperatures lead to a reduced expressivity of the gene giving an increased vitality of the mutant organisms. The gene is unable to express its action at temperatures around 14°C; the flies differ neither in their morphological characters nor in their vitality and fertility from the non-mutated flies [8]. Reactions of mutants to different temperatures are also known for *Paramaecium aurelia, Daphnia longispina, Ptychopoda seriata,* and for some other *Drosophila* mutants.

The importance of the temperature for the reaction of mutants of plant species was intensively studied by Stubbe [85], furthermore by Gustafsson and co-workers using distinct barley mutants of the Svalöf collection which were cultivated in climatically different locations of Sweden [10, 51–54, 64]. Similar trials were carried out in *Triticum durum* [2, 86]. Eight X-ray induced mutants were grown commonly with two Italian varieties and two local varieties in Italy, Tunisia, The Arabian Republic, in Syria, India and in the Lebanon. At least one of the eight mutants out-yielded the best local variety in each of these countries, but it was a different mutant in each country. The genotypes tested showed a specific reaction to the climatic and the soil conditions of the six countries demonstrating thereby their different adaptational optima.

Not only the temperature but also other environmental factors can influence the reaction of mutants in a specific way different from that of their mother varieties and of other genotypes. This holds particularly true for the photo- and thermoperiod, but also for light intensity and quality, humidity, soil quality, water supply and others. Examples on the reaction of mutants to

specific ecological conditions with many references were given by Gottschalk [14].

The problems of gene-ecology can be studied in two different ways. Distinct mutants can be grown together with their mother varieties as control material in different countries under different climatic conditions. In this way, specific responses of distinct genotypes can be discerned. It is, however, not possible to discern that particular environmental factor which is responsible for the specific reaction of the respective mutant. A prerequisite for carrying out this method is a good international cooperation. The disadvantage consists in the fact that the material is in most cases evaluated by different persons. This includes certain sources of error especially with regard to the reaction of those genes, the action of which is difficult to discern. Good examples are the behaviour of the *bif* genes for dichotomous stem bifurcation of the *Pisum* genome and certain *Pisum* genes for a weak degree of stem fasciation.

The second method consists in testing the same genotypes under different conditions of a phytotron. In this way, the influence of distinct environmental factors on different genotypes can be analysed. The same person, familiar with all the details of the material under study, evaluates the plants and the subjective error is reduced to a minimum. It should, however, be emphasized that for instance distinct short-day phytotron conditions cannot be equated with a short-day climate of a sub-tropical or a tropical country. The plant organism reacts during its ontogenetic development not only on the photoperiod and the other conditions offered in the phytotron. On the contrary, small and irregular climatic changes over short periods influence the response of the plants in a way which cannot be studied adequately in the phytotron. Another disadvantage of this method is the long duration of the trials. For many purposes, the plants are grown during their whole ontogenetic development, i.e. over a period of three to four months. It is unavoidable that such a complicated equipment gets sometimes out of function for a few hours or that the light intensity is not always constant because the lamps are abating in their efficiency and cannot be replaced so often by new ones.

We are using our comprehensive collection of *Pisum* mutants for our gene-ecological investigations. Since 1970, groups of *Pisum* mutants and recombinants were grown and comparatively evaluated in Bonn (West Germany), Zemun (Yugoslavia), Cairo (Egypt), Cape Coast (Ghana), Kampala (Uganda), Kurukshetra, Palampur, Delhi, Varanasi (N. India), Shillong (N.E. India), Udaipur, Bombay (W. India), Jabalpur (Central India), Glen Osmond (S. Australia), and Piracicaba (S.E. Brazil).

Since eight years, a phytotron is available at our institution. The main advantage of our material consists in the fact that all our mutants and almost all recombinants derive from the same mother variety and that we know their genotypic constitution. Thus, the strains differ from each other only with regard to a single allele or to the alleles of a small group of genes. In this way, it is possible to study certain interactions between distinct genes with regard to their influence on the reaction to specific environmental factors. Because of these advantages, we regard the species *Pisum sativum* as a model

plant, and there is no doubt that many of the results obtained can be generalized. In the present paper, the reaction of big groups of *Pisum* mutants and recombinants to different natural climatic conditions as well as to different phytotron conditions will be discussed. Review papers on earlier results in this field were published elsewhere [15, 18, 21, 22, 24].

2. Material and Methods

Groups of X-ray and neutron-induced mutants of the variety Dippes Gelbe Viktoria (DGV) of *Pisum sativum* were grown in Bonn and in seven countries with different climatic conditions. A great number of different recombinants, selected in the F_2 to F_4 families after having crossed different mutants with each other, were incorporated into our experiments. They are homozygous for several mutant genes and are available at the institute as pure lines, since many generations. The genotypic constitution of most of them is known to us. The material was grown in several replications per year with 30–50 plants per replication; many of the genotypes were tested in several generations. The following traits were evaluated:

1) plant height
2) flowering and ripening behaviour
3) seed production.

One hundred and twenty-six mutants and 227 different recombinants were tested under different phytotron conditions preferably in long- and short-day as follows:

Long-day	— photoperiod:	4.00 to 22.00	full light (30 klx)
		22.00 to 4.00	darkness
	— thermoperiod:	21.00 to 6.00	15°C
		6.00 to 10.00	15°C → 25°C
		10.00 to 16.00	25°C
		16.00 to 21.00	25°C → 15°C
	— humidity:	60%	
Short-day	— photoperiod:	6.00 to 18.00	full light
		18.00 to 6.00	darkness

— thermoperiod and humidity as in the long-day trials.

Fifteen seeds per genotype were sown in big Mitscherlich pots; eight to ten plants were allowed to undergo ontogenetic development. In this material, the following characters were studied:

1) plant height
2) number and length of internodes
3) number of days from sowing to flowering
4) position of the first flower and the first pod at the stem
5) seed production
6) degree of stem fasciation and stem bifurcation

With regard to the nomenclature of our material, the following regulation was used:

Only numbers: X-ray or neutron-induced mutants. Different capitals after the number (489*A*, 489*C*) designate different genotypes derived from the same irradiated embryo.

R-numbers: Recombinants selected after having crossed two mutants with each other (489*C* × 142*B* ⟶ *R* 142*C* or *R* 142*F* in F_2).

RM-numbers: Recombinants selected after having crossed a recombinant with a mutant (*R* 46*C* × 489*C* ⟶ *RM* 20*D* in F_2).

RR-numbers: Recombinants selected after having crossed two recombinants with each other (*R* 46*C* × *R* 710 ⟶ *RR* 1024 in F_2).

In the text, only the main characters of the recombinants are mentioned. In addition, they often differ from each other with regard to seed size, ripening behaviour, kind of stem fasciation among others.

3. The Behaviour of *Pisum* Mutants and Recombinants in Countries with Different Climatic Conditions

At the end of the sixties and during the seventies, gene-ecological investigations with *Pisum* mutants were carried out in the Soviet Union [80–83]. Of particular interest are the results obtained with three different groups of mutants which were comparatively grown at Novosibirsk and at a high-mountain experiment station of the Tajik SSR 2.500 m above sea level. Relative differences were found at the two locations with regard to seed production, plant height, and flowering behaviour. The yield of a chlorophyll mutant, for instance, was three to five times higher in the mountain station than in Novosibirsk; an early flowering mutant was found to be 16 days earlier at 2.500 m. These findings clearly demonstrate the relativity of the terms "useful" or "useless mutations". Similar results were obtained at two Indian locations (Kurukshetra in North India with semi-arid and Shillong in northeast India with temperate climate [65]).

3.1. THE REACTION OF MUTANTS TO CLIMATIC DIFFERENCES AT THE SAME LOCATION

The question whether mutants have in general the same adaptation optimum as their mother varieties, can already be studied at the same location provided that the material is tested over a relatively great number of generations and that there are climatic differences in subsequent years. These presuppositions are fulfilled in the moderate Middle European climate, where we often have strong differences in subsequent summers with regard to temperature and rainfall. As a consequence of these differences, the yield of our testline, Dippes Gelbe Viktoria (DGV) was found to be as twice as high in climatically favourable summers than in unfavourable ones.

The seed production of eight mutants and 12 recombinants of our *Pisum* collection, which were studied over 5-21 generations in Bonn, is graphically presented in Fig. 1. The mean values for the trait "number of seeds per plant" are related to the control values of the mother variety grown at the same location. The material can be sub-divided into two groups according to the breadth of variation of the mean values of the same genotype in different years. This variation is relatively small in the 10 genotypes of the lefthand group, whereas it is very great in the 10 genotypes of the righthand group.

Let us take mutants 26 and 189 as two characteristic examples. The plants of mutant 26 are vital dwarfs reaching stem lengths of only 10–15 cm due to

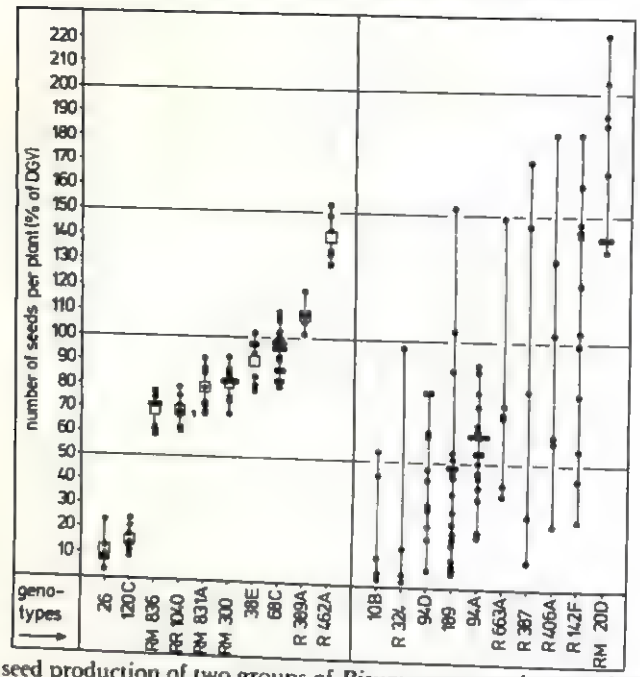

Fig. 1: The seed production of two groups of *Pisum* genotypes in several generations. Left: Relatively low variation of mean values of same genotypes in different generations. Right: Very great differences of mean values of same genotypes in different generations.

extreme shortening of the internodes. Their seed production is very low, the mean values ranging between 3 and 23% of the control values considering six generations. Thus, the mutant gene has a strongly negative selection value, which appears in each generation irrespective of the particular climatic conditions. The opposite situation is realized in recombinant *R 462A*, the last genotype of the first group. The plants of this genotype are very long and early flowering; moreover, they have *acacia* leaves (leaflets instead of tendrils, gene *tl* of the *Pisum* genome). This genotype is very productive, outyielding DGV considerably in each of the six generations tested, the mean values ranging between 129 and 153% of the control values. The deciding peculiarity of all the genotypes of the lefthand part of the graph is the fact that they show approximately the same response to the climatic conditions of Bonn as their mother variety as demonstrated by the small amount of variation of their mean values in subsequent generations. They do not markedly differ from the initial line, with regard to their climatic adaptability.

The genotypes of the righthand part of the figure show a completely different behaviour: the range of the mean values of each of them is extraordinarily broad in the generations tested in relation to the respective control values of the mother variety. Mutant 189 of this group is homozygous for at least three genes, which could be separated from each other in different recombinant types (genes for greenish flower colour, lateness and increased protein content of the seed meal). The tall and vigorous plants were found to vary considerably in their seed production in different years. In M_{17}/1976, the mean value for the character "number of seeds per plant", was only 0.16 (= 1.1% of the control value of DGV). In 1977, more than 100 plants of this mutant were grown. They were healthy and vigorous but they did not pro-

duce any seeds. The opposite situation was observed in M_{19}/1978 when the mutant surpassed its parental variety by about 50% with regard to its productivity. This behaviour demonstrates that the mutant reacts very susceptibly to the environment in a way not yet known in detail. It shows a response to a specific climatic factor completely different from the reaction of its mother variety. This holds true for all the genotypes of the righthand part of Fig. 1. They differ considerably from DGV in their adaptational optima. Some of them would certainly be very productive under distinct climatic conditions, whereas they are completely useless in countries with other climates. It is therefore not appropriate to give a general mean value for the seed production of these genotypes. It would be more reasonable to find that climate in which they are able to realize their high productivity.

3.2. THE REACTION OF MUTANTS TO THE CLIMATIC CONDITIONS OF DIFFERENT COUNTRIES

The influence of climatic factors on the reaction of distinct genes can be so strong that I was not able to discern some of my own mutants in India or Egypt. From seeds harvested from them and sown in Bonn, plants arose showing all the characteristic peculiarities of the mutants in question. This holds true for both a strengthening of the effect of the genes or their complete suppression. In the following sections, the reaction of some groups of genotypes is discussed, which are of some interest for pea breeding (early flowering genotypes and forms with stem fasciation or stem bifurcation). Moreover, the influence of environmental factors on the penetrance and the expressivity of mutant genes and on the composition of their pleiotropic patterns will be discussed.

3.2.1 The reaction of the Pisum *gene efr for earliness*

The flowering behaviour of a plant species can be evaluated by means of two different methods:

1) The number of days from sowing to the opening of the first flowers is evaluated (days to flowering); and

2) the position of the first flowers at the stem is evaluated by counting the number of vegetative or sterile nodes (node number to first flower).

It is generally assumed that the second method gives more reliable data than the first one. This has also been confirmed by our investigations. For *Pisum sativum*, the Geneva System, created by Marx in Geneva/USA, is based on the node number and the number of productive nodes. The Hobart System, created by Murfet in Hobart/Tasmania, is based on both the node number and the days to flowering. Each of the two systems has four or five classes or types in which the different genotypes can be arranged. The two systems have been compared with each other by Murfet and Marx [76]. The species shows an extraordinarily broad genetically conditioned variation of the flowering behaviour. The flowering time (days to flowering) of 747 *Pisum* lines of the Weibullsholm collection ranged between 44 and 88 days under the long-day conditions of South Sweden [1]. A considerably broader variation was observed under the short-day conditions of the north Indian winter

season. In 231 varieties and lines studied in Delhi, the number of days to flowering ranged between 38 and 145. Three varieties failed at all to flower under these climatic conditions [79]. Sharma found a positive correlation between flowering time and flowering node, but he assumes that the two characters are governed by different genes.

The genetic basis of flowering in *Pisum* is very complicated. At least for gene pairs inclusive of a series of multiple alleles are known so far to govern this process namely, E/e, Hr/hr, Lf^a/Lf/lf/lf,^{a} Sn/sn. The whole problem has been intensively discussed by Murfet [73, 74].

The X-ray induced mutant 46A of my collection is homozygous for gene *efr* for early flowering and ripening. Under West German field conditions, the first flowers are formed at the fifth to seventh node; the plants enter flowering period 7–11 days earlier than the mother variety. Following natural cross-pollination, recombinant R 46C arose homozygous for *efr* and gene *bif-1* for dichotomous stem bifurcation derived from mutant 1201A of our collection. This recombinant was used for crosses with many other mutants in order to produce *efr* recombinants of different genotypic constitution. According to Murfet [74], *efr* is identical with gene lf^a of the *Pisum* world collection. Mutant 46A and recombinant R 46C belong to the VER type of the Hobart System, whereas the mother variety, Dippes Gelbe Viktoria belongs to the L type.

The recombinant R 46C was studied in Egypt, Uganda, and at seven different locations of India with regard to its flowering behaviour. The following results were obtained:

	Days earlier than DGV
Bonn/W. Germany:	7–11
Kampala/Uganda:	7
Cairo/Egypt:	7
Bombay/W. India:	10
Varanasi/N. India:	10–13
Kurukshetra/N. India:	14–16
Udaipur/W. India:	13–19
Shillong/N.E. India:	19
Jabalpur/Central India:	19–24
Palampur/N. India:	23–30

Of particular interest is the behaviour of R 46C in Udaipur under semi-tropical conditions. In this region, crops can only be grown by means of irrigation. In 1973, there was a complete lack of the monsoon rains in this part of India, and all the genotypes tested at Udaipur inclusive of the local varieties adapted to these unfavourable conditions dried. Only R 46C survived and produced small amounts of seed [40]. This is a demonstrative example for the positive selection value of a mutant gene under extreme ecological conditions due to both a certain degree of drought tolerance and pronounced earliness enabling the plants to utilize the small amounts of water still available in the soil. Because of these peculiarities, the recombinant was incorporated into some Indian cross-breeding programmes and gene *efr* is now a constituent of a released Indian pea variety. R 46C proved to be also highly superior to DGV under the tropical conditions of Bombay,

obviously due to a certain degree of tolerance against *Sclerotium* and *Aspergillus*.

Besides R 46C, another 12 recombinants, in which *efr* is associated with different mutant genes of our collection, were studied in North India. Only four of them showed the same flowering behaviour as R 46C, demonstrating thereby that the other genes, combined with *efr*, do not influence its reaction. All the other *efr* genotypes tested, however, were considerably later, some of them even five to six weeks later than R 46C, the donor of gene *efr*:

— RM 1122B (*efr, long II*, fasciated): 37 days later (Palampur),
— RM 917 (*efr, long I*, fasciated): 38–44 days later (Palampur).

A particularly interesting behaviour shows recombinant RM 849 homozygous for *efr*, genes *sg* for reduced seed size and a gene for linear stem fasciation. This genotype was tested in Udaipur, Kurukshetra, and Palampur and showed strong differences at these locations:

	Days later than R 46C,
Udaipur:	7
Kurukshetra:	29
Palampur:	35–42

Which conclusions can be drawn from these results? The behaviour of R 46C demonstrates that gene *efr* susceptibly reacts to different climatic conditions. At most of the Indian locations tested, a positive influence of the climate on *efr* was observed, but there were striking differences in the degree of this reaction, i.e. in the degree of earliness. In Palampur in front of the Himalaya, the plants of R 46C entered flowering period almost three weeks, in Bombay only a little more than one week prior to its mother variety. The reaction of *efr*, however, depends not only an environmental factors but to an even higher extent on the genotypic background in which the gene becomes effective. The behaviour of the *efr* recombinants tested in India demonstrates that specific other mutant genes, present in their genomes, have a strongly negative effect on *efr*. The respective recombinants are genetically early due to the presence of *efr*, but in reality, they are very late. Distinct other genes suppress the positive action of *efr* partly or completely, obviously again as a response to specific climatic factors as just demonstrated for recombinant RM 849. This negative influence is probably not restricted to one single specific gene; on the contrary, several genes can act as suppressors. This problem is just under study in *efr* recombinants tested in our phytotron. The situation is already clear in the neutron-induced mutant 2590 of our collection. This is a long-stemmed early flowering double mutant. Crosses with R 46C have revealed that the earliness of 2590 is due to *efr*. In Kurukshetra, mutant 2590 was found to flower 16 days later than R 46C. In that case, only the gene for long internodes can have the delaying effect on *efr*, because no other mutant gene is present in mutant 2590. This effect, however, depends on the climate; it is not observed under the German long-day conditions.

The negative influence of distinct genes on the action of *efr* becomes not only effective with regard to the flowering behaviour of the *efr* recombinants but also to their seed ripening. In Kurukshetra, the seeds of R 46C ripened more than three weeks earlier than those of DGV. The other *efr* recombinants,

however, required a very long time for full ripening, as is evident from the following data:

	Days for seed ripening
DGV (conrol):	43
R 46C (donor of *efr*):	35
RM 849:	86
RM 879:	87
RM 878:	93
double mutant 2590:	99

These genotypes require more than double the time for ripening relative to their parental genotype R 46C, the donor of gene *efr*. Thus, the genetic advantage—the earliness—is completely annulled by other mutant genes in that part of India, with regard to both a strong delay of flowering and a strong prolongation of the ripening process.

More details on the reaction of gene *efr* on different ecological conditions and in different genotypic backgrounds are found in some former publications [15, 18, 22, 32, 40, 44–47, 49, 50, 72].

3.2.2. The reaction of fasciated genotypes

The X-ray induced mutants 107D, 123, 250A, 251A, 489C and the neutron-induced mutant 2797 show a pronounced stem fasciation. They are closely related with each other and are homozygous for more than 20 different genes, which have mutated during the irradiation of the six embryos giving rise to the mutants. Most of the mutant genes are identical in the six genotypes, but they can be distinguished from each other by small morphological differences. Only six of these genes can be discerned in the mutants; the other ones belong to a complicated system of epi- and hypostatic factors, the latter ones becoming only discernible if the former ones have been eliminated from the genomes by means of crosses [16 and recent findings]. There are two different types of stem fasciation in this material as follows:

— Strong stem fasciation.
  Uppermost internodes of the plants extremely shortened; flowers and pods therefore accumulated in the top region.
— Linear stem fasciation.
  Uppermost internodes long; flowers and pods distributed over a longer part of the stem. Very often, three or four inflorescences at the same node.

A typical representative of the first group is mutant 489C, of the second group mutant 251A. The seed production of the fasciated mutants is considerably higher than that of their mother variety due to the strongly increased number of pods per plant, as a consequence of the fasciation. They belong to the highest-yielding genotypes of our collection, when grown under the long-day conditions of Germany. Because of their complicated genetic constitution, they were used for crosses with many other mutants, giving rise to highly heterozygous F_1 hybrids. In their progenies, complicated segregations occurred. Most of the almost 700 different recombinant lines, available at our institute, arose in this way. By evaluating them, it became obvious that at least three, probably four genes for different kinds of stem fasciation are present in the fasciated mutants, only one *fasciata* gene being discernible in each of them.

The fasciated mutants were grown in six different countries and in seven climatically different regions of India; they showed a very strong response to the various climatic conditions (Table 1). In many locations, the plants of these genotypes were vigorous and healthy with pronounced stem fasciation, but they did either not flower at all or they entered the flowering period so late that no seeds could be produced. In Ghana and Uganda, the strongly fasciated mutant 489C began flowering three or even eight weeks later than the mother variety DGV and only a few seeds were produced. In South Australia, all the plants of mutant 489C died in early stages of ontogenesis, whereas the other genotypes tested showed normal development. This unusual behaviour can only be understood as a response of that particular genotype to the climatic conditions of this region. In Bonn, mutants 489C and 250A cannot be distinguished from each other. F_1 hybrids look like the parental mutants and there are no segregations in F_2. Therefore, the two mutants were regarded to be identical. This, however, is obviously not the case. In Piracicaba and Udaipur, the plants of mutant 250A did not flower, showing in general the same behaviour as mutant 489C. In Kurukshetra, however, mutant 250A flowered richly about two weeks later than DGV. Seed set was surprisingly high, about 80% of the control value, but the plants died before the seeds had fully ripened. This reaction is very different from that of mutant 489C, which did not flower at Kurukshetra, indicating that the two mutants are not identical. In the meantime, small differences between the two genotypes were also found in material tested in our phytotron.

Mutant 251A is a high-yielding form with linear stem fasciation. In contrast to 489C, the plants of this genotype flowered at most of the locations tested, but there were great differences with regard to both flowering time and seed production. In Bonn, the mutant is only a few days later than DGV. This holds also true for Udaipur and Kurukshetra, whereas flowering was considerably delayed in Palampur and in Uganda. The seed production was found to vary strongly in subsequent generations at distinct locations as follows:

—Udaipur	1972 :	105% of DGV
	1977 :	10% of DGV
—Jabalpur	1975 :	90% of DGV
	1976 :	155% of DGV
—Piracicaba	1978 :	not flowering
	1980 :	35% of DGV

In Palampur and Kampala, the yield was around 60% of the control values. Especially interesting was the behavior of this mutant in Kurukshetra. The plants flowered richly, almost simultaneously with the control plants; their pollen was normal but they were completely seed sterile from reasons not yet known.

The other three fasciated mutants considered in Table 1 were tested only at a small number of different locations. The strongly fasciated mutant 2797, morphologically identical with 489C, showed in principle the same behaviour as 489C. Mutant 123 is a linearly fasciated form similar to 251A. It showed a relatively good seed production in Palampur (80% of the control values). In Udaipur, it outyielded the mother variety considerably (140%),

Table 1: Flowering behaviour and productivity of five fasciated *Pisum* mutants in six countries and seven climatically different regions of India.

Country	489*C*	251*A*	123	250*A*	2797
Bonn/W. Germany	7-10 days later, high yield	4-6 days later, high yield	high yield	8-10 days later, high yield	8-10 days later, high yield
Cairo/Egypt	no flowers	-	-	-	-
Cape Coast/Ghana	20 days later, low yield	-	-	-	-
Kampala/Uganda	55 days later, low yield	20 days later, low yield (60%)	-	-	-
Piracicaba/N.E. Brazil	no flowers	no flowers or low yield (35%)	flowers, no seeds	no flower	no flowers or late, no seeds
Glen Osmond/S. Australia	early dying	-	-	-	-
Udaipur/W. India	no flowers or 30-40 days later; no seeds	3-7 days later, great differences in yield in 2 years	11 days later, high yield (140%)	no flowers	no flowers
Bombay/W. India	no flowers	-	-	-	-
Jabalpur/C. India	no flowers	flowering like DGV, 90% + 15% yield	-	-	extremely late, no seeds
Kurukshetra/N. India	no flowerse or extremely late, no seed	three days later, rich flowering, no seeds	-	rich flowering, two weeks later, 80% seeds, dying before ripening	-
Palampur/N. India	-	12-17 days later, 65% yield	3 days later, 80% yield	-	-
Varanasi/N. India	no flowers	-	-	-	-
Shillong/N.E. India	no flowers	-	-	-	-

showing the same behaviour as in Germany in this respect. In Piracicaba, however, the plants of this genotype flowered without producing any seeds.

All these findings demonstrate very clearly that the fasciated mutants tested react very specifically to the different climatic conditions of the various locations. In Germany, they are so productive that three commercial fodder pea varieties have been developed by means of a spontaneously arisen fasciated mutant [78]. In the African countries, in India and Brazil, they are without any agronomic value because of their non-flowering or their extreme lateness. The low productivity in these countries is due to a strongly reduced number of pods per plant as compared to Germany. Thus, one of the most characteristic peculiarities of the fasciated genotypes, their strongly increased number of pods, does not appear. Only mutants 251A, and 123 should be tested more intensively; they might be of some agronomic interest at least in distinct regions of India. This holds also true for the linearly fasciated mutant 33B with somewhat shortened internodes. In Kurukshetra, its yield was about 70% higher than that of DGV, mainly due to a strongly increased number of seeds per pod.

All the locations tested have a pronounced short-day climate. It can therefore be assumed that the photoperiod may be responsible for the abnormal flowering behaviour of the fasciated mutants. This, however, can only be studied when the material is grown under the controlled conditions of a phytotron (see Sec. 4.4.1.1).

Not only the flowering behaviour and the seed production of the fasciated mutants, but also other traits such as number and length of internodes were found to be influenced by climatic factors in a different way as compared to the control material or to other genotypes. More details are found in the papers mentioned above, furthermore in references 24, 43, 45.

In North India and in Udaipur, not only fasciated mutants but also some fasciated recombinants were tested. Some of them outyielded their mother variety considerably. This holds true for the short-stemmed recombinant RM 513, which was the only fasciated genotypes with good seed production in Palampur. Of particular interest are the recombinants R 701 and R 853 both selected after having crossed our fasciated mutant 489C with the *cochleata* mutant 5137 of Blixt's collection in Weibullsholm/Sweden. In Germany, these genotypes are linearly fasciated due to a hypostatic gene deriving from 489C, which becomes discernible in the two recombinants, because the epistatic gene for strong stem fasciation has been removed. R 701 was found to have a very high seed production in Kurukshetra. In Udaipur, the yield of R 853 was almost double as high as that of DGV due to a strong increase of both, the number of pods per plant and of seeds per pod. The stem fasciation, however, did not appear; the respective gene was unable to express its action in the semi-tropical climate of this area. The deciding point, however, is the fact that these two genotypes flowered richly in India, whereas their parental mutant 489C does not flower. It is shown in Sec. 4.4.1.2 that the non-flowering of 489C is genetically conditioned caused by gene *fis*. This gene is not present in the genomes of R 701 and R 853; it has been removed by the crosses of 489C with the *cochleata* mutant. This holds also true for recombi-

nant R 678A. The plants of this genotype began flowering in Udaipur about 25 days later than DGV, but the seeds required 16–20 days less time for full ripening. The fasciated recombinants R 142C and R 142D had in Udaipur and Kurukshetra considerably better yield than in Germany as related to their mother variety.

Udaipur has a semi-tropical climate, whereas the climate of Kurukshetra is semi-arid. Some fasciated recombinants were found to response very strikingly to these climatic differences. The plants of R 605 formed pods in Kurukshetra, but they died before seed ripening; in Udaipur, they showed normal development. All the plants of R 674A were healthy in Kurukshetra but they died before entering flowering period. In Udaipur, they were about 25 days later than DGV; they showed normal development producing low amounts of seed. Recombinant R 658D did either not flower at all in Kurukshetra or flowering began four to seven weeks later than that of DGV. The meiosis was normal, the pollen grains had a normal appearance but there was no seed production. The same behaviour was observed for recombinant R 176X in Varanasi and, as already mentioned, for the fasciated mutant 251A in Kurukshetra.

It should be noted that some fasciated recombinants required at Udaipur considerably less time for seed ripening than the mother variety DGV:

— R 142C, R 667:	11–13 days less
— R 142D, R 657, R 678A:	16–20 days less
— R 674A:	24 days less

Thus, the delayed beginning of flowering is compensated to some extent by the shortened ripening period. This also holds true for the fasciated mutants 123 and 251A.

The seed production of 26 *Pisum* mutants and recombinants, tested at Bonn and Udaipur, is given in Fig. 2. The genotyes can be classified in three groups with regard to their seed production in comparison to that of the

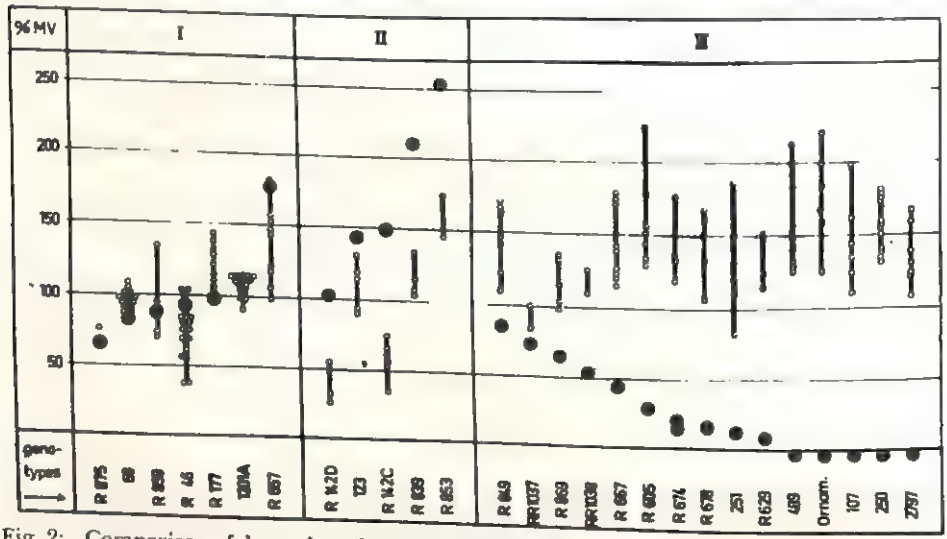

Fig. 2: Comparison of the seed production of 27 *Pisum* genotypes tested at Bonn Germany and Udaipur/India. Each dot represents the mean value for the trait, number of seeds per plant, for one generations as related to the control value of the mother variety = 100%. Open circles: Bonn values; filled circles Udaipur values.

mother vaiety. The first group shows at Udaipur approximately the same behaviour as at Bonn, indicating that these genotypes have in general the same adaptability to the altered ecological conditions as the initial line. This is not the case in the second group. These genotypes were found to be essentially more productive in Udaipur than DGV, demonstrating thereby a better adaptability to the unfavourable climatic conditions. The opposite behaviour show the genotypes of group III, whch are unable to compete with their mother variety under the climatic conditions of Western India. Many of the fasciated genotypes tested belong to this group.

3.2.3. The reduction or suppression of gene actions through environmental factors

Most of the mutant genes express their action in all the plants homozygous for them under all environmental conditions: they have a penetrance of 100%. In a small group, the penetrance can be reduced under the influence of distinct ecological factors, in extreme cases down to 0%: the action of the respective genes is not discernible. This reaction is well known for many chlorophyll deficient mutants, which often show normal chlorophyll content in warm greenhouses or hot countries (for examples and references see Gottschalk [11, 14]).

. Interesting examples for such a behaviour are two *Pisum* mutants with dichotomous stem bifurcation. Under the influence of gene *bif-1,* not the monopodial shoot structure of the species *Pisum sativum* is realized. On the contrary, two equivalent stems are formed in the top region of the plants; their stem is bifurcated. Gene *bif-2,* polymeric to *bif-1,* shows the same effect. The two genes were found to have regularly an unstable penetrance when grown in Bonn. The penetrance of *bif-1* (mutant 1201A of our collection) varied between 22% and 84%, considering more than 20 subsequent generations [12, 17, 41]. This instability appears already in the same generation at the same location. Progenies of bifurcated plants regularly consist of bifurcated and non-bifurcated individuals. If the plants of these two groups are harvested individually, bifurcated plants appear in approximately equal frequencies in the offspring of both bifurcated and non-bifurcated organisms. This means that they are genetically identical; both groups are homozygous for gene *bif-1.* The difference is not due to a segregation, but to an unstable penetrance of the *bif* gene. An environmental factor not yet known decides whether or not stem bifurcation occurs. This factor becomes already effective between neighbouring plants of the same plot.

It is difficult to interpret these findings. The apical growing point gets obviously subdivided into two independently functioning growing points, which give rise to the two stems in the top region. It is conceivable that that specific mitosis, necessary for this subdivision, is induced by a relatively low temperature, which however, can only act during a very short susceptible period. Growing points which pass through this susceptible period during the cool night hours, get the low temperature as stimulus for that particular mitosis: stem bifurcation occurs. Growing points of other plants of the same plot can pass through the susceptible period during the warm or hot daytime

and are not stimulated; stem bifurcation does not occur. The seed production of the bifurcated plants is higher than that of the non-bifurcated ones. Thus, the unstable penetrance of the *bif* genes negatively influences the breeding value of these genotypes. In spite of this disadvantage, mutant 1201A belongs to the small group of useful mutants outyielding its mother variety by 10–20% in most of the generations tested so far.

Investigations in other countries have shown that the penetrance of the *bif* genes highly depends on the climatic conditions. In Yugoslavia, it was found to be lower than in Germany. In Shillong (Northeast India), it was only 7%, in Varanasi (North India) 1%. In the other Indian locations tested (Kurukshetra, Palampur, Udaipur), all the plants of mutant 1201A were non-bifurcated; they could not be distinguished morphologically from the control plants. This holds also true for Egypt, Ghana, Uganda, and Brazil: gene *bif-1* is unable to express its action under the climatic conditions of these countries. It would thus not have been possible to select this useful mutant there. In spite of this negative reaction, mutant 1201A out-yielded the control material by about 40% at Palampur in the two generations tested so far.

The penetrance of *bif-1* depends not only on a climatic factor, but to a high degree also on the genotypic background. In some *bif* recombinants, the penetrance of the gene was found to be regularly lower than in mutant 1201A, the donor or *bif-1*; in others, it is higher. The plants of recombinant R 177, selected after having crossed 1201A with the fasciated mutant 489C, are bifurcated with fasciation below the point of bifurcation at the stem. The penetrance of *bif-1* in this genotype was found to be 95–100% not only in Germany but also in Cairo, Kampala, Udaipur, and Kurukshetra. The small number of non-bifurcated plants were weak individuals, not reaching that late stage of ontogenetic development in which the bifurcation occurs. The stabilization must be due to the influence of one of the other mutant genes present in the genome of R 177 deriving from mutant 489C. It is probably gene *sg-1* for reduced seed size, but also other genes were found to stabilize the penetrance of *bif-1*. These findings demonstrate that the positive influence of gene *sg-1* on the penetrance of *bif-1* is stronger than the negative influence of that particular climatic factor which suppresses the action of *bif-1*:

— mutant 1201A	(*bif bif*):	penetrance = 0% in India
— recombinant R 177	(*bif bif/sg sg*):	penetrance = 100% in India

This positive influence was not observed for gene *ion* of the *Pisum* genome responsible for the increase of the number of ovules per carpel (mutant 68C). Recombinant R 350, homozygous for *bif-1* and *ion*, was tested at Kurukshetra. The penetrance of *bif-1* in this combination was 0% like in mutant 1201A; nevertheless, the recombinant had a very good seed production. The total reaction of gene *bif-1* is schematically given in Fig. 3. Further details have been published elsewhere [17, 40, 43, 44, 48, 50, 72].

Some mutant genes of our collection were found to be completely unable to express their action under altered climatic conditions. This holds true for some *fasciata* genes. The plants of recombinant RM 869 show a weak but clearly discernible degree of stem fasciation with a penetrance of 100% in Germany. In Udaipur, most plants of this genotype were non-fasciated. At

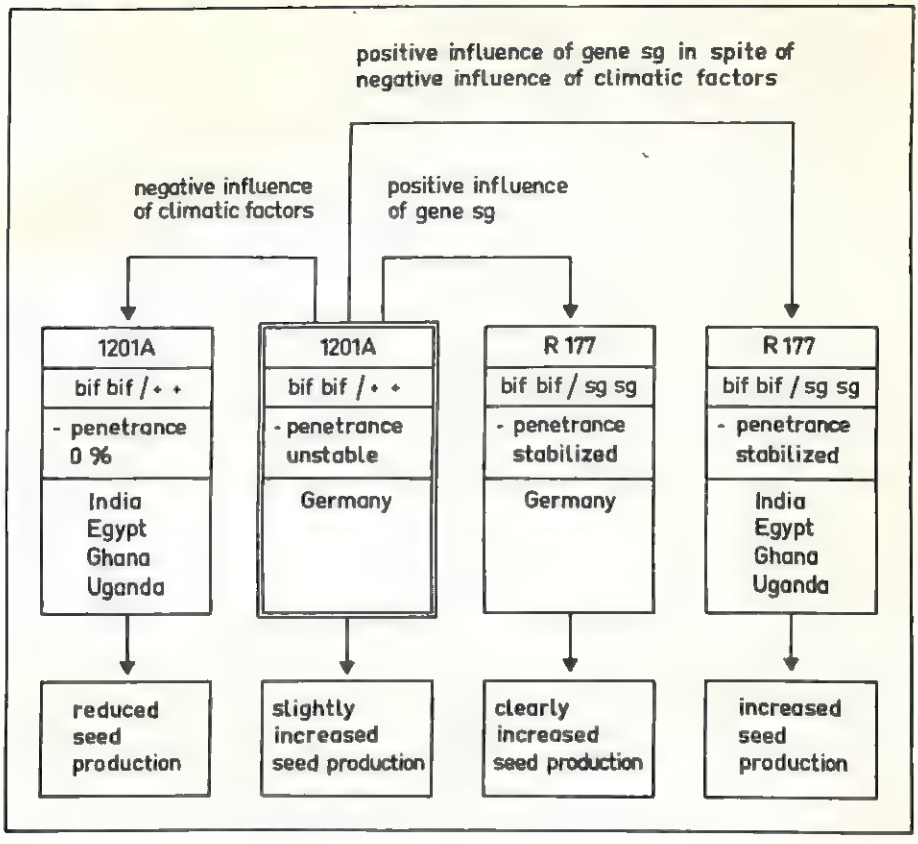

Fig. 3: The influence of climatic factors and of the genotypic background on the action of the *Pisum* gene *bif-1* for dichotomous stem bifurcation.

Kurukshetra, the penetrance of a *fasciata* gene of recombinant RM 853 was found to be 0%. The plants of R 98 did not show any stem fasciation in Kurukshetra and Varanasi when sown at normal sowing time. Sown four weeks later in Delhi, all the plants of this genotype were clearly fasciated. The environmental factor, responsible for this diverging behaviour, is probably the photoperiod. This does, however, not mean that stem fasciation in general is less pronounced in the hot Indian climate than in Germany. Seven out of the 20 different fasciated genotypes tested showed a stronger degree of fasciation at Udaipur than at Bonn [40, 44, 46].

Gene *dgl* of the *Pisum* genome causes a progressive degeneration of leaflets and stipules during ontogenesis, in that way that the leaves get brown and dry, being unable to carry out any photosynthesis. The plants of mutant 142B, the donor of *dgl*, are dainty with very low seed production. In the phytotron and in a warm greenhouse, this effect is not observed. In Cairo, the plants of this genotype could not be distinguished from the control plants of the mother variety. They were vigorous and healthy and produced normal amounts of seed. Some of their seeds were sown in Bonn and the dainty dwarfs with degenerating leaves arose. The same observations were made with

recombinants R 142C and R 142D, which contain *dgl* associated with genes for stem fasciation and for different internode lengths. They had normal leaves at Udaipur, *dgl* being completely ineffective [20, 23, 40].

A similar behaviour was observed for a spontaneously arisen *unifoliata* mutant of *Vicia faba*. The plants show a broad variation of anomalies in their flower structure, reducing their fertility strongly. In field cultivation, there are certain relations between the floral anomalies and the course of ontogenetic development: in the beginning of the flowering period, preferably abnormal flowers are formed which are unable to produce seeds. The later they are differenciated, the more normal is their structure. In the greenhouse with a permanent temperature of 25°C, the plants had the abnormal leaves, but completely normal flowers with full fertility. Also in this case, the mutant gene can express its negative action only below a distinct temperature threshold [21].

The examples just given demonstrate that distinct environmental factors can decide whether mutants are fully fertile having a good selection value or whether their fertility is so reduced that they have no phylogenetic chances. In exceptional cases, even the alternative viability or lethality is governed by environmental factors. Mutant 11A of our *Pisum* collection is a long-living lethal mutant. Under field conditions, a gradient with regard to the chlorophyll formation is observed, the lowest leaves having the normal amount of chlorophyll, whereas the upper ones are almost chlorophyll-free. The plants die after having formed 7–10 leaves. The lethality, however, occurs only under the normal conditions of insolation. Grown in dark shadow, the chlorophyll content is normal and the lethality can be overcome [11, 23]. The mutant is obviously identical with the *albina-terminalis* mutant described by Lamprecht [66].

A similar effect of the temperature is observed in mutant 227A of our collection. Under German field conditions, the plants show a strong chlorophyll deficiency of the variegate type followed by lethality. In the greenhouse, avoiding low temperature and high light intensity, the plants developed normally, producing many flowers. They were, however, sterile because of manifold meiotic irregularities [13]. The mutant is obviously homozygous for two genes. The chlorophyll gene is a lethal factor which becomes effective in early stages of ontogenetic development. If the negative action of this gene is suppressed by a distinct environmental factor, the second gene becomes effective causing sterility.

3.2.4. The alteration of pleiotropic patterns under the influence of climatic factors

Almost all mutant genes of higher plants are pleiotropic, the composition of their pleiotropic patterns being a strong handicap for the agronomic utilization of many prospective genotypes. In exceptional cases, such a pattern can be influenced by environmental factors positively or negatively. Gene *ion* of the *Pisum* genome (mutant 68C) causes an increase of the number of ovules per carpel of about 30%, resulting in an increase of the number of seeds per pod. This positive trait, however, is accompanied by the

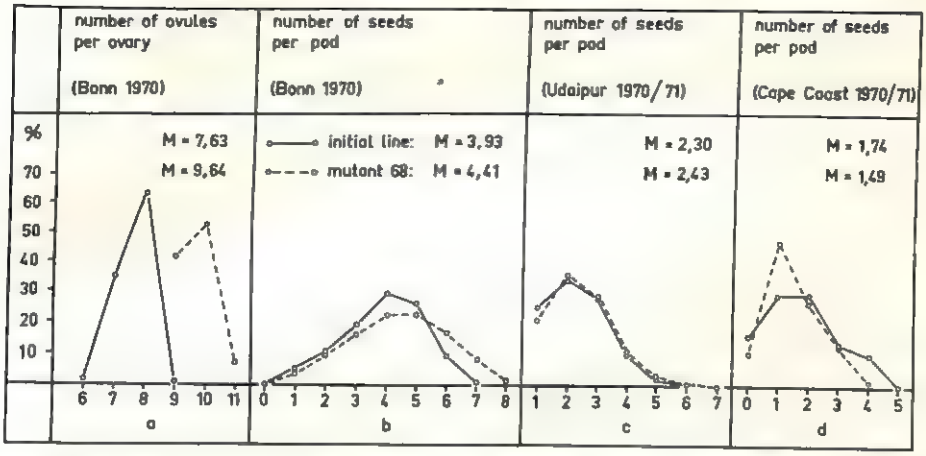

Fig. 4: The reaction of the *Pisum* gene *ion* for increased number of ovules per carpel to the climatic conditions of Germany, Western India, and Ghana (explanation in the text).

reduction of the number of pods per plant. Thus, the pleiotropic pattern of this gene consists of a positive and a negative component, which regularly appeared in each generation tested in Bonn.

The expressivity of gene *ion* was found to depend to some extent on climatic conditions, but in a different way for the two components of the pattern (Fig. 4). In Udaipur, the number of seeds per pod of mutant 68C and of its mother variety was considerably lower than in Germany and there was practically no difference between the two genotypes with regard to this trait. Thus, gene *ion* was unable to express its favourable action in the semi-tropical climate of this part of India. In Ghana, the fertility within the pod was even stronger reduced, but in 68C to a higher extent than in the control material. In contrast to Germany, the mean value of 68C was lower than that of DGV. It would thus not have been possible to select this useful mutant at the two locations mentioned. Particularly interesting, however, was the behaviour of the mutant in Kurukshetra (Fig. 5). The number of seeds per pod was increased like in Germany, but also the number of pods was considerably increased due to strong branching of the plants, which was not observed in the mother variety. Thus, the positive part of the pleiotropic pattern of gene *ion* appeared and the negative part was altered positively, under the semi-arid climate of this part of India, resulting in a strong increase of the seed production [19, 43, 44, 47]. If we summarize the reaction of gene *ion* with regard to its pleiotropic pattern at five locations tested, the following behaviour was observed:

Bonn, Kampala:	increased number of seeds per pod decreased number of pods per plant
Udaipur:	number of seeds per pod as in DGV decreased number of pods per plant
Cape Coast:	decreased number of seeds per pod decreased number of pods per plant
Kurukshetra:	increased number of seeds per pod increased number of pods per plant

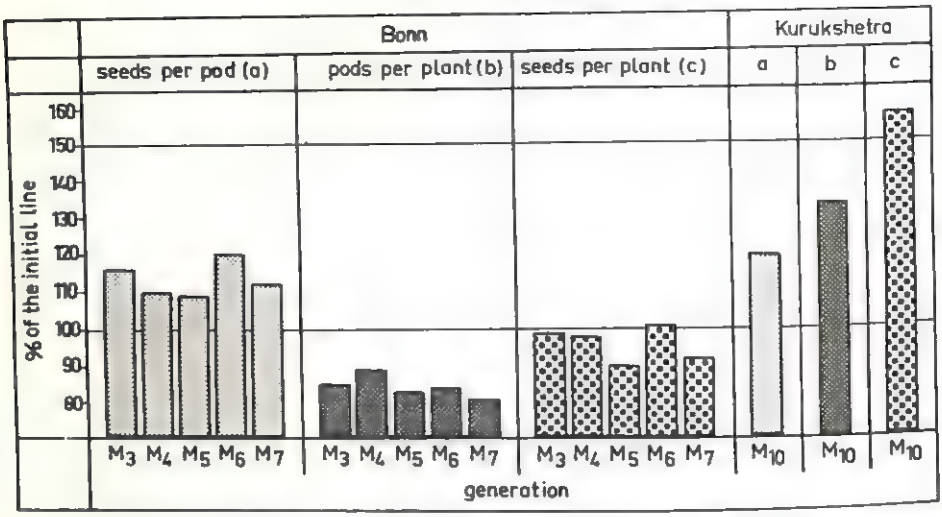

Fig. 5: Comparison of the yielding characters of the *Pisum* mutant 68C homozygous for gene *ion* when grown in Bonn/Germany and Kurukshetra/North India. In Kurukshetra, the pleiotropic pattern of the gene is altered positively.

Thus, the mutant is without any agronomic value in western India and Ghana. It might be of some interest in cross-breeding programmes in Germany and it is a very promising genotype in North India.

4. The Flowering Behaviour of *Pisum* Mutants and Recombinants under Controlled Phytotron Conditions

The reaction of different genotypes to specific ecological factors can reliably be analysed only in the phytotron. Only a single environmental factor is altered in different trials, whereas all the other conditions remain unchanged. Any differences in the behaviour of the genotypes tested must therefore be due to that altered factor.

Investigations of this kind were already carried out by Brücher in the 40s [3, 4], who cultivated *Antirrhinum* mutants under different temperature, light and humidity conditions in small climate cabinets. In different artificial climates, several mutants were found to be superior to their initial lines, and the selection value of the genotypes tested varied from climate to climate. Very intensive gene-ecological investigations were carried out by Gustafsson and co-workers with barley mutants using the Stockholm phytotron. Four commercial varieties, developed from induced mutants, and a great number of hybrid combinations were tested commonly with their parental varieties. Up to 30 different climatic conditions were offered to the material and up to 14 different traits were evaluated. Moreover, a group of early flowering genotypes, an *erectoides* and an *eceriferum* mutant were incorporated into the experiments. The reactions of mutants and mother varieties were very different, particularly with regard to the photo- and thermoperiods offered [5–7, 55–63].

Since 1980, a phytotron is available at our institute. So far, 126 mutants and 227 recombinants of our *Pisum* collection were studied with regard to

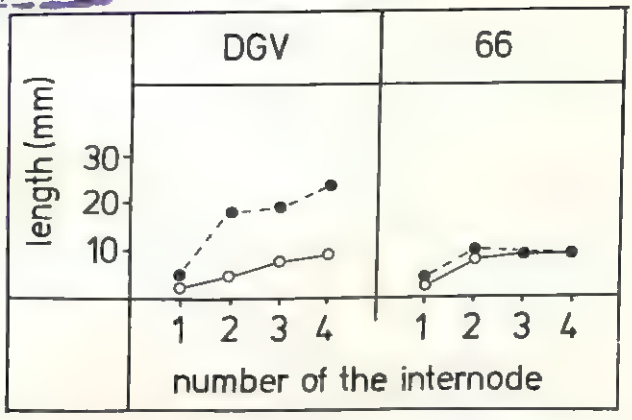

Fig. 6: The response of the short-stemmed *Pisum* mutant 66 and its mother variety to different light intensities. Solid line: 12,000 lux; broken line; 600 lux.

their reaction to different temperatures, light intensities, photo- and thermoperiods. All the values obtained were related to the control values of the mother variety Dippes Gelbe Viktoria (DGV)

4.1. INFLUENCE OF THE LIGHT INTENSITY

Already in the 70s, preliminary gene-ecological studies with some of our *Pisum* mutants were carried out in a small climate box in which the material could be investigated only in very early stages of ontogenetic development. The mutants were grown in two tiers within the box, the upper tier being exposed to a light intensity of 12,000 lux, whereas the lower tier received only 600 lux [42, 71]. As expected, not only the mother variety but also most of the mutants tested showed a strong etiolation under the conditions of 600 lux, resulting in a considerable increase of the internode length and in general weakness. The X-ray induced mutant 66, however, being characterized by shortened internodes in the upper part of the stem, did not show this reaction (Fig. 6). No differences in the internode lengths were observed between the plants grown in full light and those grown under strongly reduced amounts of light. The same behaviour was found for the fasciated mutant 489C. These genotypes clearly show a high degree of tolerance to low amounts of light and would thus have a selection advantage under these unfavourable conditions. The flower mutant 94A showed the opposite behaviour: the degree of etiolation was under weak-light conditions stronger than in the control material. Clear differences were also found with regard to the chlorophyll formation as follows:

mutant 489C in 12,000 lux :	10% less chlorophyll than DGV
in 600 lux :	30% more chlorophyll than DGV
mutant 94C in 12,000 lux :	15% more chlorophyll than DGV
in 600 lux :	20% less chlorophyll than DGV

Thus, the two genotypes react completely different to the two light conditions.

4.2. INFLUENCE OF THE TEMPERATURE

In order to learn whether there is a genetically conditioned variation with

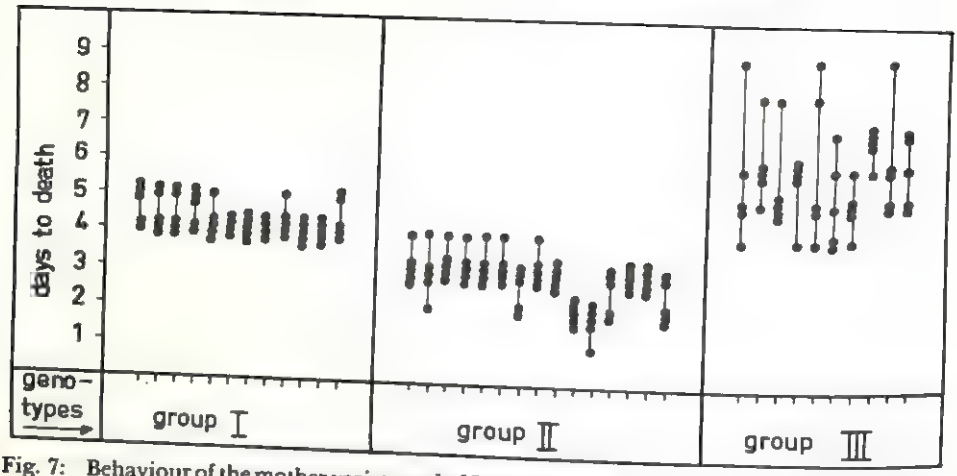

Fig. 7: Behaviour of the mother variety and of 36 *Pisum* mutants in the phytotron at 40°C. Each dot gives the value for one plant; does connected by vertical lines belong to the same genotype.

regard to heat tolerance in our *Pisum* material, 126 mutants were tested in the phytotron under the following conditions:

Germination and early development in long-day 16/8 hours
15°C during night, 25°C during daytime in order to obtain healthy and vigorous plantlets.
20 days after sowing at constant 30°C
25 days after sowing at constant 35°C
30 days after sowing at constant 40°C

This high temperature was maintained till the plants died.

All the genotypes tested survived the phytotron conditions at 35°C, whereas none of them was able to tolerate 40°C. There were, however, clear differences with regard to the time of onset to death (Fig. 7). The mother variety and most of the mutants survived under 40°C only for a period of four to five days (group I of the graph). The genotypes of group II showed a greater sensitivity to this high temperature, the plants dying already during the first two or three days after germination. The genotypes of group III, on the other hand, showed a certain degree of tolerance, being able to survive longer under these unfavourable conditions [25].

The purpose of this experiment was to preselect genotypes which might have certain selection advantages in countries with hot climates. The mutants of group III shall be tested under more realistic phytotron conditions of a hot climate similar to the winter conditions of North India. Later on, the preselected genotypes will be tested on a larger scale in semi-tropical or semi-arid regions. In this way, it might be possible to discern the breadth of ecological adaptability of our material with regard to this climatic factor and to incorporate some prospective genotypes into breeding programmes, which have not yet been utilized because of lack of information.

Certain indications have already been obtained in a phytotron experiment with the following conditions:

short-day 12/12 hours
thermoperiod: 10.00 to 17.00 30°C
17.00 to 22.00 30°C → 20°C

	22.00 to 24.00	20°C → 10°C
	0.00 to 6.00	10°C
	6.00 to 10.00	10°C → 30°C
humidity:		40%

Thirty mutants, eight recombinants and the mother variety were tested; the findings obtained were very diverging. The evaluations had to be made prematurely because of the bad condition of most of the genotypes, obviously due to the high temperature, but there were striking differences between the genotypes. In some mutants, 50% of the plants or even more died before entering flowering period. Some others, however, were completely healthy, demonstrating thereby a high degree of tolerance to these unfavourable conditions. This holds particularly true for some genotypes homozygous for gene *efr* for earliness. The plants of mutant 45C and recombinants R 46C, RM 836 and RM 837 of this group produced even small amounts of seed. This positive reaction is in agreement with the behaviour of gene *efr* in India. It was already mentioned that mutant 46A, the donor of *efr*, proved to be relatively insensitive to the hot climate of Western and Central India and that the gene has been used for developing an Indian commercial variety. The two *efr* recombinants RM 20D and RM 20E were completely healthy in the phytotron, but they had only tiny flower buds which did not undergo any further development as a reaction to both the high temperature and the short-day (see below). Mutants 37B and 2323 were healthy but very late; only small flower buds had been formed when the trial was stopped. The plants of a short-stemmed *afila* recombinant were likewise completely healthy, but they remain vegetative under short-day conditions.

The flowering behaviour of some genotypes, grown under the conditions mentioned above (10–30–10°C), is compared in Fig. 8 with that of plants grown under a thermoperiod of 15–25–15°C. It becomes clear that the higher temperature during daytime has a strong influence on the speed of ontogenetic development. The difference between the two trials is especially great in the early flowering waxless recombinant RM 837. The short-stemmed

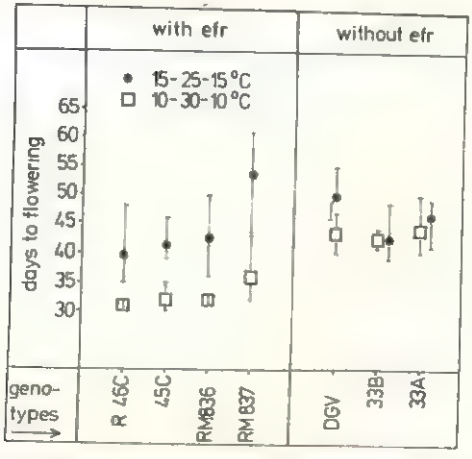

Fig. 8: Comparison of the flowering behaviour of seven *Pisum* genotypes under short-day phytotron conditions with two different thermoperiods.

fasciated mutants 33A and 33B, on the other hand, show no or only small differences in their reaction to the two thermoperiods.

It was already mentioned that the chlorophyll deficiency of distinct mutants is often not discernible in countries with hot climates. It is very probable that the temperature is that climatic factor which suppresses the action of the respective genes, but not yet evident. A similar case was analysed in one of our *Pisum* mutants. The plants of mutant 159 are almost chlorophyll-free and lethal when grown in the field. The chlorophyll deficiency appeared also in the greenhouse, but the plants did not die. On the contrary, lateral branches developed showing only a small degree of chlorophyll deficiency. After decapitation of the main stem, flowering and fruiting stems developed from these lateral branches. Thus, the lethal effect of the mutant gene could completely be blocked through appropriate environmental conditions. The cultivation of homozygous material of this mutant in a climate chamber revealed that the chlorophyll formation depends on the temperature. Plants cultivated at 15°C were unable to produce chlorophyll. When these plants were transferred into a chamber with 25°C, the basic leaves remained chlorophyll-free, but the newly developing ones had chlorophyll. The opposite effect was observed when normal green plants, which had been cultivated at 25°C, were transferred to a chamber with 15°C. The chlorophyll already present remained unchanged, but the new leaves were chlorophyll-free. Further investigations have shown that the threshold is 17°C, in that particular genotype: temperatures above 17°C allow chlorophyll synthesis, those below 17°C suppress it [11].

4.3. INFLUENCE OF THE THERMOPERIOD

Preliminary investigations have shown that some mutants are influenced in their physiological activities through the thermoperiod in a way different from that of their mother variety. The neutron induced mutant 2906, having big leaves at the stalks of the inflorescences, did not show any chlorophyll deficiency when grown under short-day conditions 12/12 hours, in combination with a continuous temperature of 20°C. A thermoperiod of 20/5°C, led to a considerable reduction of chlorophyll synthesis. The chlorophyll content of the plants was only 66% of the control value of the mother variety [70].

In order to study the response of different genotypes to different temperature conditions, 17 mutants and 20 recombinants of our collection were commonly grown with DGV in the phytotron under the following three regimes:

trial 1 : continuous temperature of 12.5°C
trial 2 : continuous temperature of 25.5°C
trial 3 : thermoperiod of 12.5°C during night and 25.5°C during daytime

The material was grown in long-day 18/6 hours, in all the three trials; humidity was 60%. The aim of these experiments was to study the influence of different temperatures, preferably on the flowering behaviour of the genotypes.

The flowering behaviour of eight recombinants, homozygous for gene *efr* for earliness in combination with other mutant genes, is presented in Fig. 9.

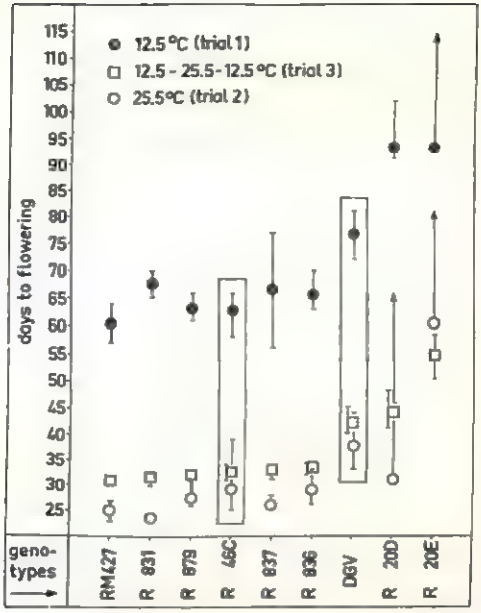

Fig. 9: The flowering behaviour of the mother variety DGV and of eight recombinant lines containing gene *efr* for earliness and other mutant genes under three different phytotron conditions. Recombinant R 46C is the donor of gene *efr*. Each dot gives the mean value of 10 plants.

The genotypes are ordered according to their behaviour under normal phytotron conditions (12.5°C during night, 25.5°C during daytime). Under the constantly given low temperature of 12.5°C (trial 1), the control plants of DGV needed 77 days for reaching the flowering period. This was 39 days more than under the permanently given high temperature of 25.5°C (trial 2). This behaviour was generally observed not only in the genotypes of Figs. 9 to 11 but also in the other genotypes tested, but clear differences between some of them and the mother variety were found. The mean values obtained for the same genotypes in trials 2 and 3 lay closely together demonstrating thereby that the influence of the low night temperature of trial 3 has only little influence on the ontogenetic development of the plants, provided that a higher temperature during daytime is available. The permanently given low temperature, however, causes the strong delay of the plant growth expected.

Of particular interest is the reaction of the recombinants RM 20D and RM 20E, not only in the temperature but also in the photoperiodic trials. They were selected after having crossed the fasciated mutant 489C with the early flowering recombinant R 46C. Their genotypic constitution is as follows:

RM 20D: *efr* for earliness (from R 46C)
bif-1 for dichotomous stem bifurcation (from R 46C)
ccr for reduced chlorophyll content (from 489C)
long III for very long internodes (from 489C)

RM 20E: *efr* (from R 46C)
bif-1 (from R 46C)
short II for shortened internodes (from 489C)

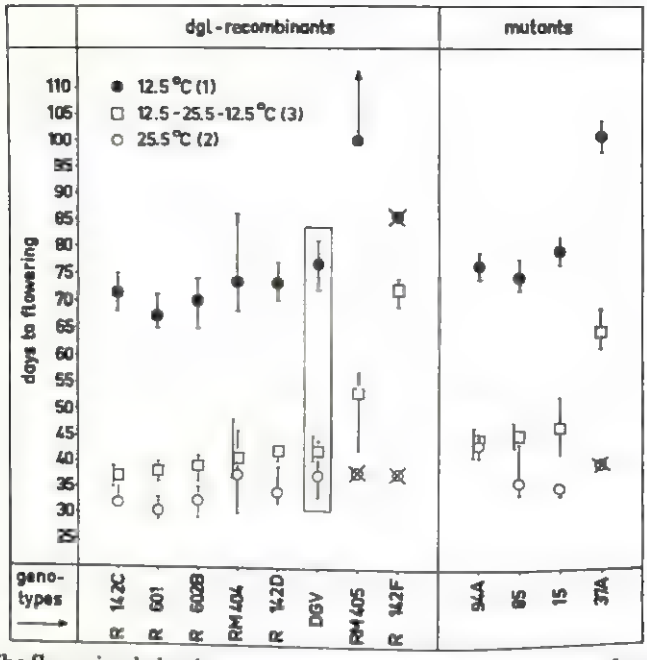

Fig. 10: Left: The flowering behaviour of seven recombinants homozygous for gene *dgl* for leaf degeneration in combination with other mutant genes in the phytotron.
Right: The behaviour of four different mutants under the same conditions.

When we compare the flowering behaviour of these genotypes with that of R 46C, the donor of gene *efr*, the following findings were obtained:

RM 20D, trial 1: 30 days later than R 46C
trial 2: considerably later than R 46C*
trial 3: 11 days later than R 46C
RM 20E, trial 1: considerably later than R 46C*
trial 2: considerably later than R 46C*
trial 3: 22 days later than R 46C

In the trials marked by asterisks, the plants of the two genotypes showed a very heterogeneous behaviour. The first plants entered flowering period as drawn in the graph, the last ones had not yet flowered when the trials were stopped. Therefore, no mean values for the trait, number of days of flowering, can be given. The characteristic peculiarity of these two genotypes is a strong delay of flowering, which appeared in RM 20E much more pronounced than in RM 20D. Induction of flower formation occurred in these plants at the normal stage of ontogenetic development, but only tiny flower buds were produced at the lower nodes of the stem and these did not undergo further development. Fully developed functionable flowers were only formed considerably later at higher nodes. This behaviour was not observed in R 46C and in the other *efr* recombinants studied in these experiments; it can therefore not be a reaction of gene *efr* as such. On the contrary, one of the additional mutant genes present in the genomes of RM 20D and RM 20E influences *efr* negatively, in that way the gene cannot express its full action, its earliness. It will be shown later that these two genotypes react also very susceptible to different photoperiods with regard to their flowering behaviour (Sec. 4.4.1.1).

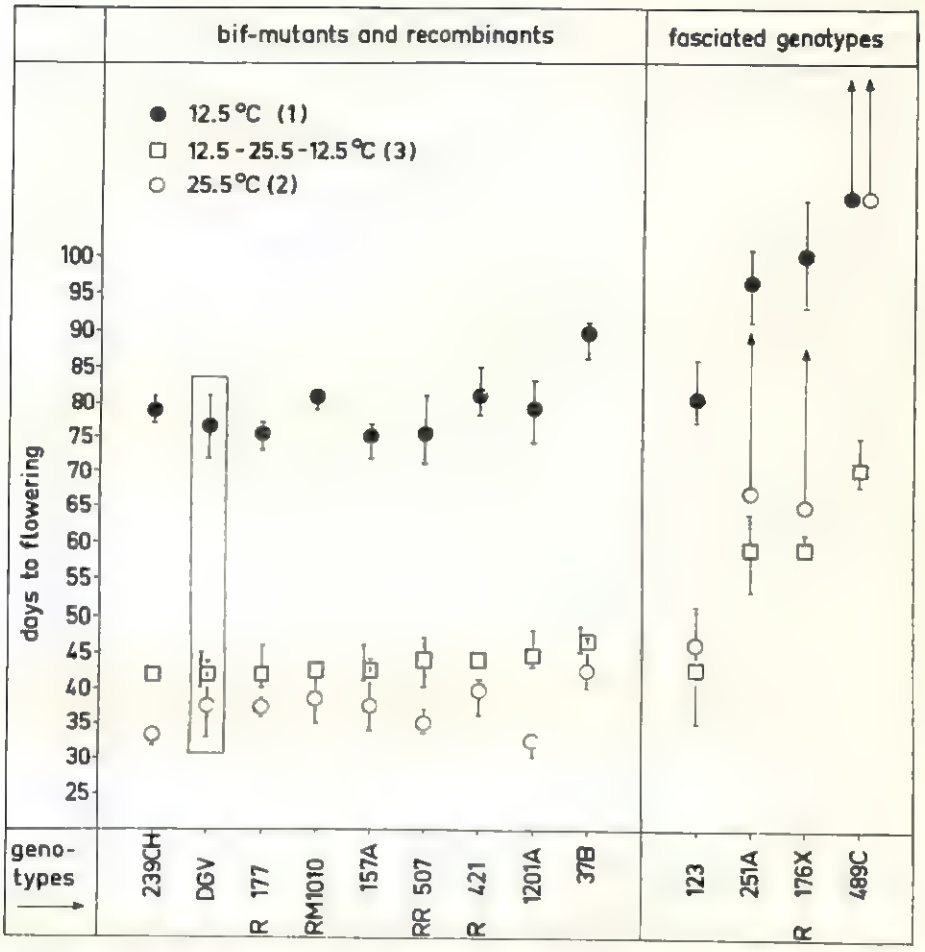

Fig. 11: The flowering behaviour of eight genotypes with dichotomous stem bifurcation and of four genotypes with stem fasciation under three different phytotron conditions.

In the lefthand part of Fig. 10, the behaviour of seven different recombinants, homozygous for gene *dgl* for leave degeneration in combination with other mutant genes, is graphically presented. The plants of most of these genotypes flowered earlier than the mother variety in all the three phytotron trials. This holds, however, not true for recombinants RM 405 and R 142F. Full flowering of RM 405 occurred only in trials 1 and 3, but considerably later than in all the other genotypes of this group. In trial 2 (permanent temperature of 25.5°C), only tiny flower buds were formed, none of which showing any further development, although the plants were tall and vigorous. A particularly interesting behaviour, however, was observed in recombinant R 142F. There was no induction of flower formation at all in any of the tall and healthy plants of this genotype in trials 1 and 2, i.e. under constant low or high temperatures. The plants remained in a vegetative state until the experiments were stopped. In trial 3, floweing occurred. Thus, the plants of this genotype need the daily change of low and higher temperatures for being

able to enter flowering period. These findings show that one of the mutant genes, present in the genome of recombinant R 142F, specifically controls the reaction of the plants to the temperature conditions.

An especially unexpected reaction to the temperatures offered was observed for some fasciated genotypes of our collection (Fig. 11, righthand part). Under the normal phytotron conditions of trial 3, mutant 123 began flowering almost simultaneously with the control plants, whereas the other three genotypes were considerably later. In contrast to all the other mutants and recombinants studied, these four genotypes needed under the continuously given high temperature of 25.5°C, more time for reaching the flowering period than under the thermoperiod of 12.5–25.5–12.5°C. Thus, the constant temperature of 25.5°C negatively influences the flowering behaviour of these fasciated genotypes, whereas it has a positive influence on the other genotypes tested.

The reaction of further mutants and recombinants to the three different temperatures is illustrated in Figs. 10 and 11; the whole problem has been discussed by Gottschalk [36]. Different response of some *Pisum* genotypes to a constantly given temperature of 20°C and to a thermoperiod of 20/5°C was also observed with regard to the internode length [70].

4.4 THE INFLUENCE OF THE PHOTOPERIOD

Investigations on the photoperiodic reaction of different *Pisum* genotypes were carried out by Marx [67–69]. He found striking differences of the reaction of distinct varieties to long- and short- days and used these results for classifying the genotypes as follows:

K type: late in long-day,
a modest quantitative delay of flowering in short-day
G type: late in long-day,
a dramatic, almost qualitative delay in short-day
I type: insensitive to differences in the daylength

A Turkish accession of *Pisum sativum* ssp. *syriacum* was medium late in long-day, flowering around node 15; in short-day 15/9 hours, some plants of this genotype remained vegetative for over 90 nodes. According to his results, a polygenic system controls both nodes to flower and degree of the response to daylength. Some pea mutants were cultivated in the Soviet Union in the field under three different photoperiods with around 18, 8 and only 4 hr of light. The control plants and two mutants showed the positive correlation between daylength and beginning of flowering expected: the longer the photoperiod, the earlier the flowering. A third mutant, however, was found to react indifferently to the photoperiod: flowering time and number of sterile nodes were approximately equal in the three different photoperiodic conditions [84].

A great number of mutants and recombinants of our *Pisum* collection were comparatively studied under long- and short-day phytotron conditions (18/6 hours or 12/12 horus, respectively). The behaviour of some of them was furthermore investigated at continuous light and in extreme short-day, with only 6 hours light. It was furthermore tried to compare the long-day, phytotron results with the long-day field results obtained in West Germany and the short-day phytotron results with the short-day field results obtained in India.

4.4.1. The comparison of long- and short-day phytotron results

The main aim of most of our phytotron experiments was to compare the reaction of a great number of mutants and recombinants to the long- and short-day conditions mentioned in Chapter 2. In the present paper, only a review on the results can be given; more details have been published in former papers [22, 24, 26–33, 35, 37, 39]. Preferably two groups will be discussed, namely early flowering genotypes and genotypes with apical stem fasciation. They are of interest for both problems of basic research and of mutation breeding.

4.4.1.1. The behaviour of early flowering genotypes: The recessive gene *efr* of our *Pisum* collection is of direct value for pea breeding, because it causes a pronounced degree of earliness. According to crossing experiments carried out by Murfet [74], it is identical with gene *lf*^{a} of the *Pisum* world collection. Mutant 46A and recombinant R 46C, the donors of *efr*, were crossed with many other mutants of our collection giving rise to a great number of different recombinants available at the institute as pure lines. Seventy-three of them were tested in the phytotron with regard to flowering behaviour and stem structure. The flowering behaviour of most of them is illustrated in Figs. 12 and 13.

The comprehensive material is ordered in Fig. 12 according to the number of days to flowering under long-day phytotron conditions (18/6 hours). Only the mean values are given in order not to burden the graph with too many details. The variation of the single values per genotype is considered in Fig. 13. The plants of the mother variety DGV began flowering in long-day 38 to 46 days after sowing, the mean value being 41.8 days. The corresponding values of recombinant R 46C were 29.6 days for the mean with a variation from 28 to 33 days. Thus, R 46C flowered around 12 days earlier than the control material. Figure 12 shows that many of the *efr* recombinants tested showed in principle the same flowering behaviour as R 46C demonstrating thereby that the other mutant genes present in their genomes do not influence

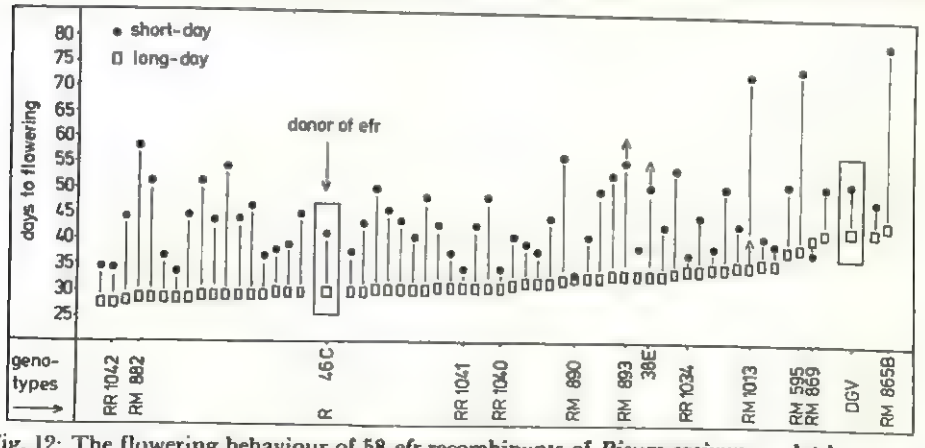

Fig. 12: The flowering behaviour of 58 *efr* recombinants of *Pisum sativum* under long- and short-day phytotron conditions (18/6 hours or 12/12 hours, respectively). The mean values are compared with those of R 46C, the donor of gene *efr* for earlines, and with control values of the mother variety, Dipped Gelbe Viktoria (DGV).

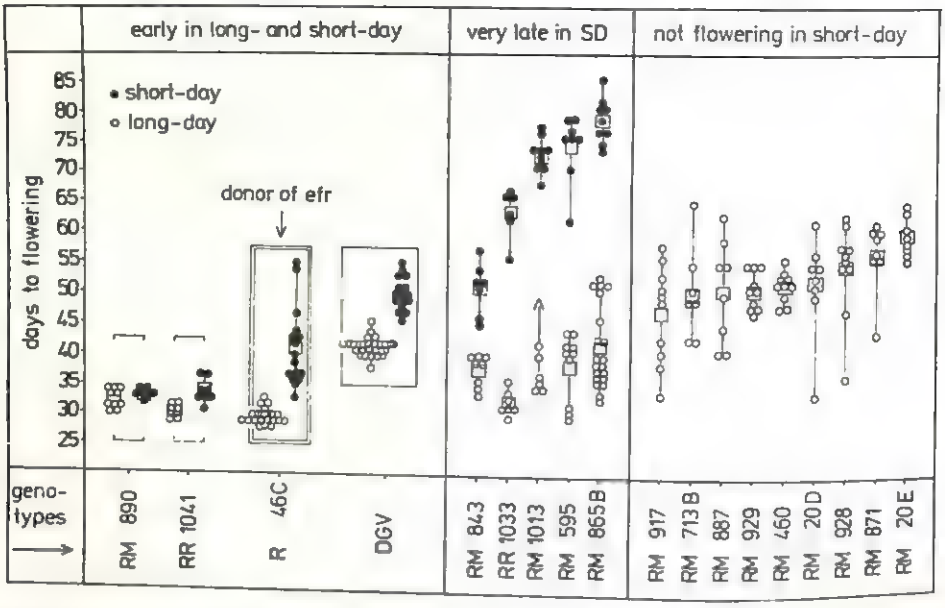

Fig. 13: The flowering behaviour of 16 *efr* recombinants under long- and short-day phytotron conditions as compared with R 46C, the donor of gene *efr* for earliness, and with the mother variety DGV. Each dot gives the value for one plant; the squares are the mean values for the respective genotypes.

efr with regard to its action. A few genotypes flowered two days earlier than R 46C (RM 451, RR 1042), possibly indicating a positive effect of one of their mutant genes on *efr*. Quite a number of *efr* recombinants, however, showed the opposite behaviour, i.e. they began flowering later, or even considerably later. This holds particularly true for nine genotypes which do not produce functionable flowers under short-day phytotron conditions (Fig 13). Some examples may elucidate this situation (long-day 18/6 hours):

RR	1038	:	6 days later than R 46C
RM	595	:	9 days later than R 46C
RM	869	:	11 days later than R 46C
RM	865B	:	14 days later than R 46C
R	460	:	22 days later than R 46C
R	928A	:	25 days later than R 46C
RM	20E	:	30 days later than R 46C

Further details can be seen from the two graphs. This delay can only be understood as a negative effect of other mutant genes on *efr*, which are present in the genomes of these recombinants. The initiation of flowering, i.e. the trasition from the vegetative to the reproductive state of the plants, occurs in these genotypes at the same stage of ontogenetic development as in R 46C, but only minute flower buds are formed at the lower nodes of the stem, unable to develop into normal flowers. These are produced only at higher nodes resulting in the delay of flowering observed. A similar behaviour was already mentioned for some *efr* recombinants as a reaction to high temperatures.

Let us now compare the reaction of the same genotypes to the two different photoperiods. It is clear that the short-day plants will need a longer time for

reaching the flowering period than the long-day plants, and one should expect a certain parallelism of the mean values obtained in the two trials. Figure 12, however, demonstrates that this parallelism is not realized. On the contrary, some genotypes showed only very small differences between the long- and short-day means (RR 1041, RR 1040, RR 1034), whereas very great differences were found in most of the recombinants tested (RM 882, RM 1013, RM 595, RM 865B and many others). The delay of the short-day plants is due to the fact that fully developed flowers are only formed at relatively high nodes of the stem, whereas the lower nodes have exclusively the minute floral buds already mentioned. This regularity appears already in R 46C, but it is realized in other *efr* recombinants in a much higher, and in distinct *efr* genotypes also in a much lower extent. This diverging behaviour can only be due to differences in the genotypic constitution of the various *efr* recombinants tested. Distinct mutant genes or gene groups intensify the tendency of R 46C to produce non-functionable minute buds in short-day, whereas other mutant genes reduce this tendency.

A special situation in this respect is realized in recombinants RM 890 and RM 869, which have the following genotypic constitution:

RM 890 (from 107D ×R 46C)
- short II for strongly shortened internodes (from 107D)
- weakly fasciated stem (from 107D)
- smaller narrower leaves (from 107D
- *efr* (from R 46C)

RM 869 (from 250A × R 46C)
- long II for long internodes (from 250A)
- linearly fasciated stem (from 250A)
- ccr for reduced chlorophyll content (from 250A)
- *efr* (from R 46C)

The plants of RM 890 showed in short-day the same flowering behaviour as in long-day (Fig. 13), those of RM 869 were in short-day even earlier than in long-day.

The whole *etr* material studied can be subdivided into the following three groups:

Group I : ± early in long- and short-day
Group II : early or medium late in long-day, very late in short-day
Group III : late in long-day, not flowering in short-day

Representatives of these three groups are given in Fig. 13. Of particular interest are the genotypes of group III. The initiation of flower formation occurs normally, but only the tiny buds mentioned above are formed in short-day. When long-day was given to these short-day plants, the tiny flower buds already present remained unchanged but from the new buds normal flowers arose. This happened in all the 15 recombinants of this group demonstrating thereby, that these genotypes react specifically to the photoperiod. As this behaviour is not observed in R 46C and in many other *efr* recombinants, the action of a specific mutant gene must be postulated, which is responsible for the suppression of *efr* in short-day, whereas it has a reduced degree of effectiveness in long-day. This gene has provisionally been designated as *fds* (flower development suppressor [32]). It is not present in the genome of R 46C and it annulls the positive effect of *efr* completely. When the plants of R 46C

had already fully ripened seeds in the short-day trials, not a single normal developed flower had been produced in all the *efr* recombinants of group III. So far, 15 *efr* genotypes of our collection were found to show this short-day reaction. Six of them have not yet been studied in long-day, the other ones are considered in the righthand part of Fig. 13. The 15 genotypes just mentioned do not have a discernible specific gene in common; thus, we did not yet succeed in identifying gene *fds*. It has obviously no visible effect on plant morphology and becomes only discernible under distinct photoperiodic conditions. This is an interesting example for complicated interactions between different mutant genes, as well as between gene *fds* and distinct environmental factors.

4.4.1.2. The behaviour of fasciated and of some other genotypes: It was already mentioned in section 3.2.2, that some of our high-yielding fasciated mutants do either not flower at all in countries with short-day climate or that they are so late that the plants die before seed ripening. It seems to be obvious that these genotypes show a specific reaction to the photoperiod different from the reaction of their mother variety and of the other genotypes tested. In order to prove this hypothesis, not only the fasciated mutants but also many fasciated recombinants derived from them were tested in the phytotron under long- and short-day conditions (Figs 14–16).

The strongly fasciated mutants 107D, 250A, 489C and 2797A, which have been used for producing many fasciated recombinants, were found to flower more than four weeks later than the control material under the long-day phytotron conditions offered to them. Reliable mean values cannot be given, because not all the plants of these genotypes had entered flowering period when the trials were terminated. The linearly fasciated mutant 251A was not so late; mutant 123 of this group began flowering only a few days later than DGV (Fig. 14). The plants of 107D and 123 flowered richly, whereas the other mutants produced only a small number of fully developed flowers and had many undeveloped buds. In short-day, mutant 123 began flowering three weeks later than in long-day. The other five fasciated mutants of this group, however, did not flower at all. They were healthy and vigorous but their apical growing points formed exclusively minute leaves. When the phytotron was changed from short- to long-day, all the plants of these five fasciated mutants had flower buds eight to ten days later. This is not only a confirmation of our findings obtained in India, Egypt, and Brazil, but it is the evidence that a mutant gene controls the photoperiodic reaction of these mutants. They need long-day for the initiation of flower formation. The gene in question has been provisionally designated as *fis* (flower initiation suppressor [22, 27]). The same behaviour was observed for the fodder pea variety Ornamenta, and for the fasciated mutant VI-10 of Mrs. Vassileva's collection in Sofia, and also for the recombinant R 629 of our collection selected after having crossed mutant 489C with the short-stemmed mutant 66. The plants are very long and linearly fasciated and have gene *fis*; all the three genes derive from mutant 489C.

The flowering behaviour of most of the fasciated recombinants considered

in the middle part of Fig. 14 is similar to that of the control material. Recombinant R 657 is insofar of interest as the two photoperiods used did not influence the flowering behaviour differently; exactly the same mean values were obtained for the long-and short-day plants. It should be mentioned that the two X-ray induced fasciated mutants 33A and 33B flowered in long- as well as in short-day with only small differences of their mean values in the two trials. They do not contain gene *fis* in their genomes.

We have used our phytotron for preselecting fasciated recombinants which flower in short-day. Some of them are considered in the middle part of Fig. 14. The flowering behaviour of another 33 fasciated recombinant strains under short-day phytotron conditions is given Fig. 15. Quite a number of them was found to flower simultaneously with the mother variety or even earlier. Recombinant R 729B seems to be a promising genotype. It was selected after having crossed mutant 489C with the cochleata mutant 5137 of Blixt's collection and has the following genotypic constitution:

long III for very long internodes (a gene hypostatic in 489C)
pronounced stem fasciation and bifurcation (from 489C)
earlier than 489C (a gene hypostatic in 489C)

The plants flowered richly in short-day simultaneously with DGV. Gene *fis* has been eliminated through the hybridization. The seed production in the phytotron was considerably better than that of the mother variety (60% more). Another promising genotype is RM 27 from the cross 489C × R 46C. The tall fasciated plants flowered in short-day, 10 days prior to DGV and had a considerably better seed production [30, 37]. Some of the recombinants considered in Fig. 14 and 15 should be tested in countries with short-day climates, not only with regard to their flowering behaviour but also to their yielding potentialities. In this way, it might be possible to utilize the favourable yielding properties of fasciated peas to some extent, in those countries in which the garden pea is used for closing the protein gap.

All the genotypes just mentioned do not contain gene *fis*, it has been

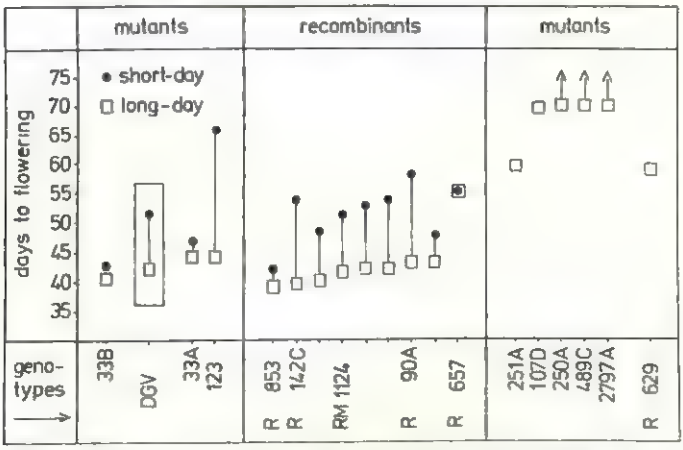

Fig. 14: The flowering behaviour of eight fasciated *Pisum* mutants and of 10 fasciated recombinants under long-and short-day phytotron conditions. The six genotypes of the third group did not produce any flowers in short-day.

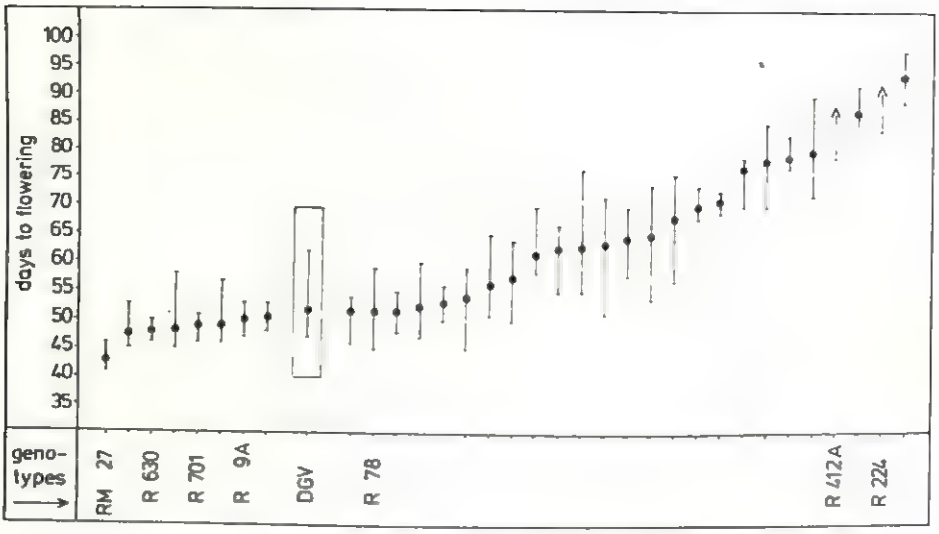

Fig. 15: The flowering behaviour of 33 fasciated *Pisum* recombinants under short-day phytotron conditions. Some of them flower as early as or even earlier than the mother variety DGV. The genotypes considered in the graph were not yet tested in long-day.

removed through the crosses. Another 22 fasciated recombinants were found to be extremely late in short-day. They had only minute flower buds when the trials were terminated. Many plants of these genotypes died before having produced any buds. The initiation of flower formation had obviously occurred, but these genotypes are so late that there are no chances for any seed production. They are as useless for agronomic purposes as the *fis* genotypes. All the plants of another five fasciated recombinants were still in a vegetative state when the trials were stopped. Their apical growing points formed only small leaves and no floral buds, thus showing the same behaviour as mutant 489C and related forms, obviously due to the presence of gene *fis*.

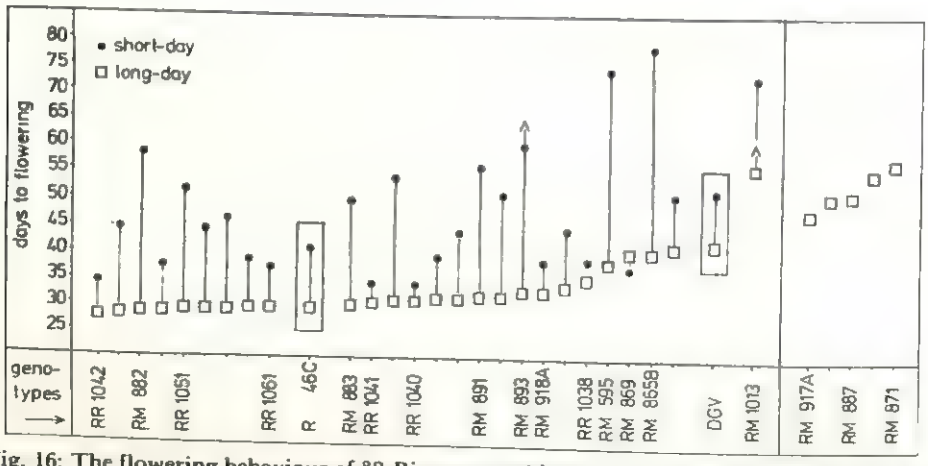

Fig. 16: The flowering behaviour of 32 *Pisum* recombinants, homozygous for a gene for stem fasciation in combination with gene *efr* for earliness and different other mutant genes, under long- and short-day phytotron conditions. The five recombinants in the right-hand part of the graph are unable to produce functionable flowers in short day.

It should be emphasized that the genes *fis* and *fds* are completely different in their action:

Gene *fis* suppresses flower formation in short-day completely or it delays it so strongly that the plants die before seeds can be produced.

Gene *fds* specifically influences gene *efr* so negatively that no functionable flowers are produced in short-day. Flower initiation occurs, but the development of the buds to flowers does not take place.

The combination of *fasciata* genes with gene *efr* should theoretically result in high-yielding early flowering plants. Thirty-one recombinant lines of our collection have this constitution in combination with other genes or gene groups. Their phytotron behaviour is graphically illustrated in Fig. 16. Many of these genotypes showed at least in long-day the same flowering behaviour as R 46C, the donor of gene *efr*. In short-day, they differ strongly from each other, but some of them seem to be promising in this concern (RR 1038, RR 1040, RR 1041, RR 1042, RR 1061). They are not only earlier than DGV, but even earlier than R 46C and should be tested in countries with short-day climate. The strongly diverging reaction of the same genotype to the two different photoperiods, which was already demonstrated in Fig. 13 and 14, clearly shows again that the genotypic constitution of a mutant or a recombinant is the deciding factor in this respect. A small group of fasciated *efr* recombinants did not produce any fully developed flowers in short-day; they had only the minute floral buds as a consequence of the action of gene *fds*.

Besides the fasciated mutants mentioned above, also the nonfasciated mutant 37C was fount to be unable to flower in short-day. This is a micromutant which cannot be classified reliably in segregating families in the field. The plants are a few days later and had a somewhat higher yield in all generations tested so far. Under long-day phytotron conditions, they were four weeks later than DGV and had a strongly increased number of shortened internodes as follows:

DGV: 18.8 internodes
3.4 cm mean internode length
37 C: 32.0 internodes
2.6 cm mean internode length

In two short-day trials, 14 plants of this mutant were still in the vegetative state 93 days after sowing, when the trial was terminated. Two plants produced very late some abortive flower buds. With regard to the stem structure, the following mean values were obtained:

DGV: 24.9 internodes
4.0 cm mean internode length
37 C: 28.2 internodes
2.3 cm mean internode length

Thus, the two genotypes reacted very differently to the two photoperiods. It is not sure whether the mutant contains gene *fis*. It is possible that the plants would have flowered if they would have been cultivated even longer [28].

A very unusual behaviour was found for recombinant R 142F selected after having crossed mutant 489C with the almost semi-lethal leaf mutant 142B. In

the field, the non-fasciated tall plants begin flowering more than two weeks later than DGV. Their seed production varied considerably in subsequent generations, obviously due to a strong susceptibility to environmental factors. In two long-day phytotron trials, the plants began flowering 25 or 26 days later than DGV; in two short-day trials, all the plants of R 142F remained vegetative. They showed the same behaviour as the plants of mutant 489C in the same trials. Nine weeks after sowing, the phytotron conditions were changed from short- to long-day; at that time, the control material had already well developed pods. Three weeks later, mutant 489C had flower buds as a long-day reaction. Even after five weeks of long-day influence, no sign of flower formation was observed in R 142F. This reaction is obviously due to the presence of gene *fis* derived from 489C, which shows an even stronger effect in the genome of R 142F than in 489C [35]. The recombinant was also tested in the phytotron in Hobart, Tasmania. Only two plants were grown in 8 hours short-day. They flowered at nodes 64 and 76(!); the latest plant took 165 days to flower [75]. The discrepancy between the Bonn and Hobart results are probably due to differences in the light quality.

4.4.2. The reaction of Pisum *genotypes to continuous light*

Twenty-six mutants and 41 recombinants of our *Pisum* collection were grown at continuous light in the phytotron. Most of the genotypes tested showed either no or only small differences in their flowering reaction to permanent light or to long-day 18/6 hours. That means that an additional amount of light beyond 18 hours per day has only little effect on the transition from the vegetative to the reproductive stage of the plants. This held particularly true for most of the *efr* recombinants and for the mother variety. Greater differences were observed in some late flowering genotypes, which began flowering at continuous light considerably earlier than in long-day (Fig. 18):

the micromutant 37C eight days earlier
the fasciated mutants 251A and 489C nine or more than 14 days earlier, respectively

The seed production of many genotypes was found to be poorer in permanent light than in long-day. This is also valid for the mother variety and for recombinant R 46C, the donor of gene *efr* for earliness. Some fasciated mutants and recombinants, however, outyielded the control material considerably at continuous light. Recombinant RM 1122B, selected from cross 489C × R 46C, is particularly notable in this respect. In the field, RM 1122B produced a 34 to 76% higher yield than DGV in five subsequent generations. In the permanent light trials, however, the recombinant outyielded both DGV and R 46C by about 500%. The plants have the following genotypic constitution:

long II for long internodes (a gene hypostatic in 489C)
fasciated and bifurcated (from 489C)
ccr for reduced chlorophyll content (from 489C)
efr for earliness (from R 46C)

More details on the reaction of our *Pisum* genotypes to permanent light were published elsewhere [33, 34].

Many genotypes were repeatedly tested under the same phytotron condi-

tions with continuous light and a high degree of conformity of the mean values was obtained. This, however, was not the case in a very small group of recombinants which showed relatively great differences in their flowering behaviour in subsequent trials. Interestingly, the respective genotypes (RM 20D, RM 20E, R 142F) showed also under other phytotron conditions strongly diverging reactions in comparison to other genotypes, as already discussed in the various sections of the present article. They react obviously very drastically to insignificant changes of the phytotron conditions. These genotypes will be studied more in detail in order to clarify the causes of their inconstancy.

4.4.3. The reaction of Pisum *genotypes to extreme short-day conditions*

Three mutants and 29 recombinants were grown in the phytotron under extreme short-day conditions, with only 6 hours light per day. All the other environmental conditions were the same as in the short- and long-day trials (25°C during daytime, 15°C during night, 60% humidity [38]).

Only three of the 20 control plants of DGV reached the flowering stage, all the others died in earlier stages of ontogenetic development as a consequence of the insufficient amount of light. Two mutants and five recombinants showed a similar behaviour. In another group of six recombinants, flower buds appeared, but did not develop further. The fasciated mutant 489C and recombinant R 142F were still in a purely vegetative state when the trial was terminated 83 days after sowing. At that time, some *efr* genotypes had already fully ripened seeds.

Out of the 33 genotypes tested, only 17 flowered but there were some losses in most of the genotypes cultivated. The flowering behaviour of these genotypes is illustrated in Fig. 17. The material is subdivided into two groups

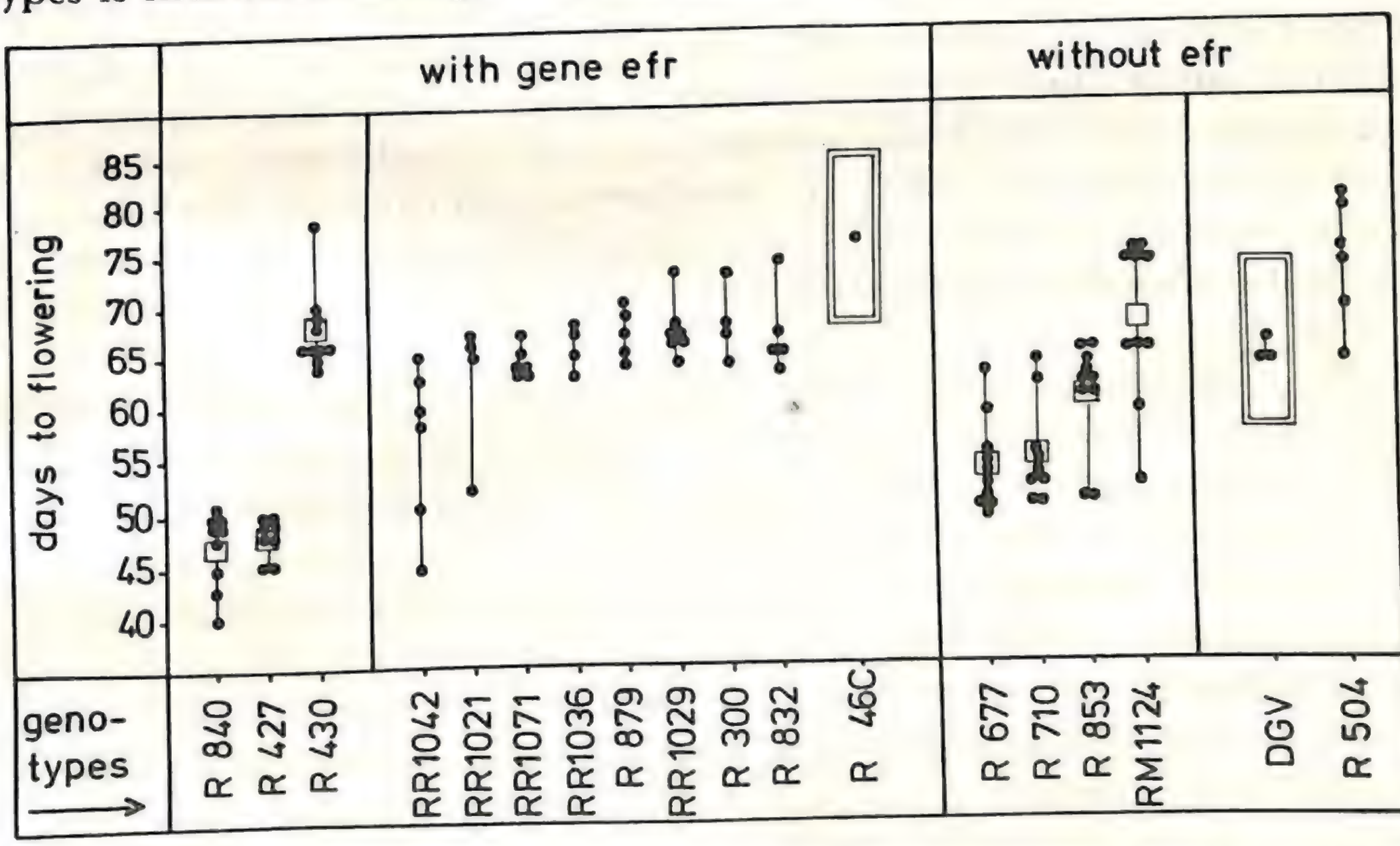

Fig. 17: The flowering behaviour of 17 *Pisum* recombinants under extreme short-day conditions in the phytotron. The genotypes of the first group contain gene *efr* for earliness derived from R 46C. The genotypes of the second group do not contain this gene. Each dot gives the value for one plant. DGV (Dippes Gelbe Viktoria) is the mother variety of our radiation genetic experiments.

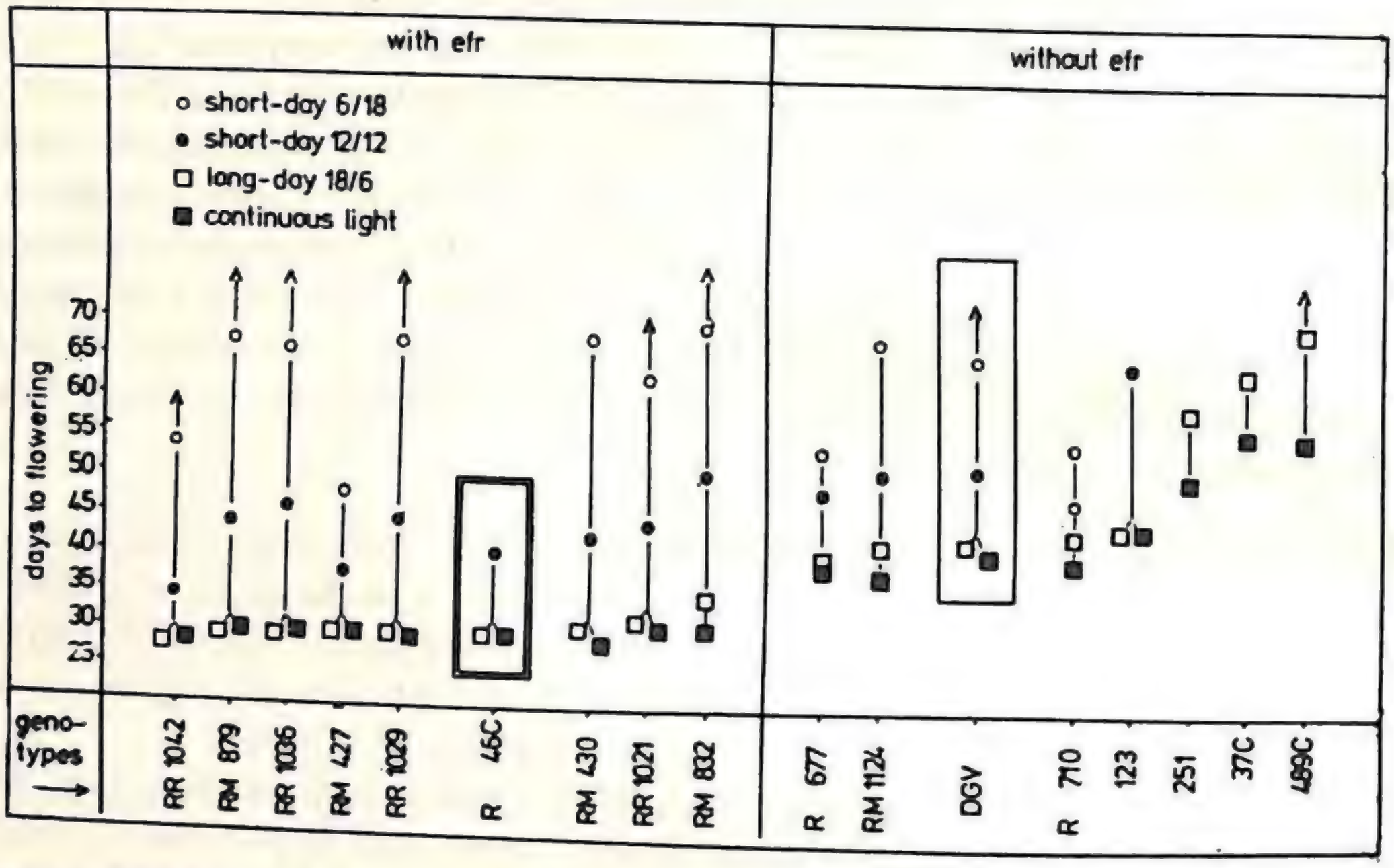

Fig. 18: The flowering behaviour of four *Pisum* mutants, 12 recombinants and the mother variety under four different photoperiods in the phytotron. Each point represents the mean value for the respective genotype. R 46C is the donor of gene *efr* for earliness.

according to the presence or absence of gene *ear* for earliness. Recombinant R 46C, the donor of this gene, reacted as negatively as its mother variety to the unfavourable conditions. Out of the 10 plants originally present, only a single plant formed some normal flowers, whereas the others had undeveloped buds or they died in earlier stages. A completely different behaviour was observed for the recombinants RM 840, RM 427, and RM 430. Their development was relatively normal and they produced small amounts of seed, thus demonstrating a high degree of tolerance to these highly unfavourable photoperiodic conditions. From another eight *efr* recombinants, some plants flowered but they were weak and died before seed ripening. Therefore, no mean values are given for them in the graph. A more or less normal flowering showed also some fasciated recombinants considered in the righthand part of the figure.

If we compare the results of the 6/18 hours and 12/12 hours short-day trials, it becomes clear that—as expected—a considerably longer time is required to reach the flowering stage in extreme short-day than in normal short-day (Fig. 18, Table 2). It is surprising that a very broad variation of the number of days to flowering within the same genotype in the same trial was observed in all the phytotron experiments, although the plants developed under equal environmental conditions. This demonstrates the unreliability of this trait, which is not found in the characters, node with the first flower, and number of internodes. Of particular interest is the flowering behaviour of the short-stemmed *efr* recombinant RM 430, because of its lateness. Gene *efr* for earliness cannot express its action under the negative influence of a distinct other mutant gene present in RM 430. This negative reaction appeared especially strongly in the 6/18 hours experiment (Table 2).

Table 2: Comparison of some plant characters of seven *Pisum* recombinant lines grown at normal and extreme short-day phytotron conditions (Upper group: *efr* genotypes; lower group: fasciated genotypes)

Genotype	short-day 12/12 hours				short-day 6/18 hours			
	Days to flowering	Plant height (% of DGV)	Number of internodes	Node with first flower	Days to flowering	Plant height (% of DGV)	Number of internodes	Node with first flower
RM 840	32-36	144.0	14.5	9.9	40-51	152.1	15.1	12.8
RM 427	32-42	84.1	21.6	11.3	45-50	92.6	20.4	15.9
RM 430	38-48	64.5	17.8	10.5	64-78	109.8	21.5	20.4
R 853	39-44	173.9	21.1	17.3	50-65	219.1	22.6	20.8
R 710	45-51	173.4	23.7	22.2	50-64	193.4	22.6	21.2
R 677	45-55	138.4	19.6	17.8	49-63	166.7	19.2	17.7
RM 1124	45-49	118.4	20.2	19.2	52-75	189.5	24.2	22.0

The influence of the photoperiod on the flowering behaviour becomes particularly clear in Fig. 18. Seventeen genotypes of our collection are considered in the graph, with regard to their flowering in four different photoperiods. From mutant 123 and recombinant R 46C, only three mean values are given, because these genotypes did not flower in 6/18 hours short-day. Mutants 37C, 251A and 489C do not flower in both normal short-day 12/12 hours and extreme short-day 6/18 hours. The graph demonstrates the small differences in the reaction to continuous light and long-day 18/6 hours of most of the genotypes tested. The differences between long-day 19/6 hours and short-day 12/12 hours, however, are very pronounced. This holds also true for the differences between short-day 12/12 hours and short-day 6/18 hours. Some genotypes had very similar mean values in the four photoperiodic trials (RM 879, RR 1036, RM 430, RR 1021, RM 832, DGV). Others, however, showed a very specific behaviour (RM 427, R 677, R 710), not agreeing with the recombinants just mentioned.

4.4.4. The influence of the photoperiod on the position of the first flower and the number and length of internodes

In all the 22 phytotron trials carried out so far, not only the flowering time but also the position of the first flower at the stem and the number and length of the internodes were recorded. It is not possible to discuss the numerous data available in detail, in the present article, but some examples may demonstrate in which way the traits just mentioned are influenced through different photoperiods.

It was just shown that the beginning of flowering is the more delayed, the less light per day is available to the plants. In most of the genotypes studied, the number of sterile nodes increases with decreasing amounts of light. The recombinants R 46C, RM 430 and RR 1021 are given in Fig. 19 as representatives of this type of reaction. The correlation appears, often not clearly, if the conditions of continuous light are compared with those of long-day 18/6 hours. Thus, the respective genotyes show in principle the same behaviour for both characters, the days to flowering and the position of the first flowers at the stem. In the two different short-day trials, however, this regularity is very clear (Fig. 19, middle part). In the same genotypes, a negative correlation between number of internodes and amount of light per day was observed: the less the light, the higher the number of internodes per plant (righthand part of Fig. 19). Thus, the reduction of the amount of light available for photosynthesis has three effects on the mutants and recombinants of this type:

1) The plants produce more internodes,
2) they enter flowering period later, not only with regard to the number of days to flowering but also to the position of the first flowers at the stem, and therefore
3) the transition from the vegetative to the reproductive stage occurs ontogenetically, the later the less light is offered to the plants (Type A). The degree of this reaction to the various photoperiods strongly differs between the genotypes tested.

A small group of genotypes does not follow this scheme. Recombinant R

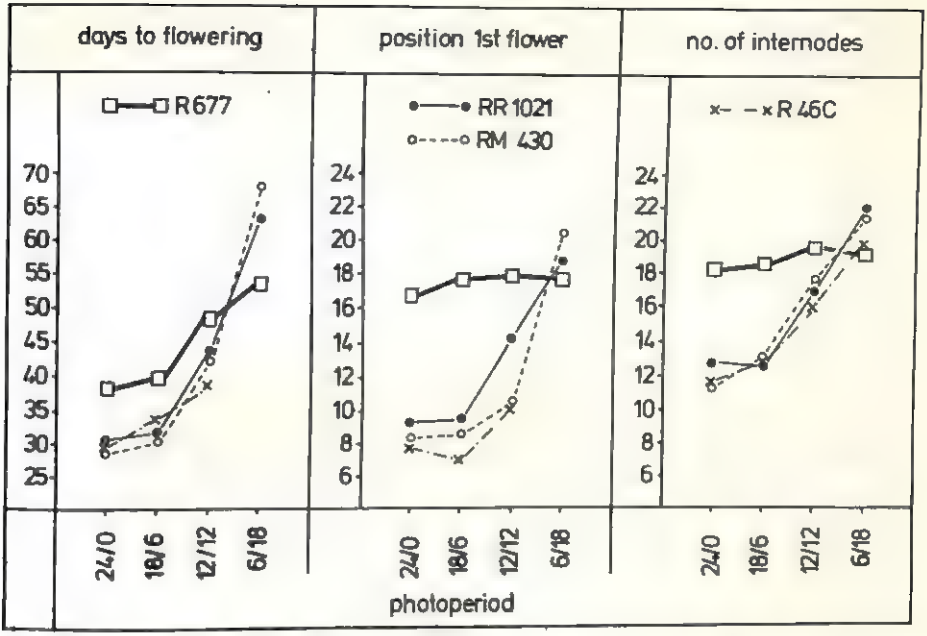

Fig. 19: The influence of the photoperiod on the number of days to flowering, position of the first flower at the stem and the number of internodes per plant.

677 is given in Fig. 19 as a representative of this second type (Type B). The recombinant was selected from the cross 489C × Blixt's *cochleata* 5137 and has the following genotypic constitution:

- *long I* for longer internodes (a gene epistatic in 489C)
- very weak stem fasciation (a gene hypostatic in 489C)
- earlier than the two parental mutants (a gene hypostatic in 489C)

With the regard to the flowering time, the same negative correlation as in Type A was observed in R 677, but the differences of the mean values in the four photoperiods were less pronounced. In the other two characters studied, however, no specific reactions to the four different photoperiods occurred; the mean values lay closely together and did not show any significant correlation to the different amounts of light.

We had expected to find also a negative correlation between internode length and amount of light, especially in the 6/18 hours trial as an indication of a certain degree of etiolation as a consequence of the low amount of light. This, however, was not the case in most of the genotypes tested. The plants were considerably longer in the 6/18 hours trial as compared to the 12/12 hours trials. This increase, however, was not due to the elongation of the internode length but to the increase of the number of internodes per plant as demonstrated in Fig. 19. More details on the reactions just discussed have been published in former papers [29, 39].

5. Concluding Remarks

The main result of our gene-ecological investigations, carried out since more than 15 years with around 350 different genotypes of our *Pisum* collection, is

the fact that almost each genotype shows its specific reaction to distinct ecological conditions. The response of the same mutants or recombinants to different climatic or phytotron conditions is so broad and often so unexpected that no predictions can be made: each genotype has to be tested under as many different environmental conditions as possible. Only in this way, the true breadth of the ecological adaptability of the respective genotypes becomes discernible, and there is no doubt that many mutants of many crops, existing since decades in our collections, could be utilized agronomically if their real potential of adaptation to climate, soil, and water supply could be known.

The study of prospective genotypes in the phytotron is a useful way for making certain preselections. It can, however, not replace a close international cooperation between those geneticists, who have developed comprehensive collections of mutants and recombinants and those colleagues who want to utilize some of them in specific climates. It is very easy to select high-yielding mutants of a long-day species which flower under short-day phytotron conditions. This, however, does not mean that these genotypes are really suited for a cultivation in a specific short-day climate. Some of our high-yielding fasciated pea genotypes, for instance, were found to be unable to express their pronounced stem fasciation in India. Thus, they have lost that particular character which is the presupposition for their high seed production; they are useless. Other genotypes were found to be abnormally long in short-day phytotron experiments, whereas they were strongly shortened under the influence of distinct Indian short-day conditions.

The photoperiod is only one climatic factor, the temperature and the thermoperiod are others, and it is impossible to test a great number of mutants in the phytotron under exactly the same climatic conditions existing in specific regions of other countries or continents. We can analyze the influence of single climatic factors on our genotypes in the phytotron, but we are unable to test them under the compound conditions of whole different climates. This becomes already clear if we compare the long-day phytotron results with the findings obtained with the same genotypes under West German long-day field conditions. Essentially greater are the differences between our short-day phytotron findings and the field results obtained in countries with short-day climates [22, 29, 35, 50]. Thus, a phytotron is a very useful equipment for analyzing specific reactions of distinct genes to specific environmental factors, but for practical purposes, the direct test of the preselected genotypes under the field conditions of a given country is irrevocable. This is being done with groups of our *Pisum* genotypes in India and Egypt and has already led to encouraging results as shown in the present paper.

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2

Genome Organization in Plant Cells: Is Repetitive DNA Redundant?

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1. Introduction

Recent knowledge of molecular biology tells us that the amount of DNA present in the cell exceeds by far that needed for encoding all its proteins [33, 47]. As a result, it is now more difficult than ever to answer the questions: "To what purpose this lavishness will serve? Will redundant DNA be of practical use or become a nuisance? If this redundancy has no function compatible with clear thinking, perhaps, it has a unrecognized mission?"

There are always straightforward answers, some correct. One here is that the major portion is regulator, the remaining coding. However, the great quantitative differences in the non-coding DNA between species, even genera, of eukaryotes, which are evolutionary successes, support the view that most of the non-coding part of DNA is of no use to the vital activities of the cell. In illustration, we refer to a case observed among cereals. The self-pollinating species of the genus *Lolium*, say *L. temulentum*, contains about 40% more DNA than the cross-pollinator *L. perenne*. The great proportion of additional DNA in *L. temulentum* is represented by repetitive sequences [41]. The nucleus of *Festuca drymeja* concentrates 50% more DNA than that of *F. scariosa* [68]. Besides these, there are numerous other no less obvious examples. The whole course of the evidence seems to be consistent with the idea that there exists ultimately selfish DNA [15, 59] indolent to the extent to take care only of its own good (see [95–97]).

The aim of this review is not to take any side on this controversial issue. Moreover, the idea of selfish DNA does not appear to be amenable to direct experimental verification [16]. This review is rather intended as a synopsis of the data in the literature pertaining to the non-coding part of DNA. Our data on the non-coding part of DNA were obtained with the cereal genome. These data will be considered along with those under review.

2. Organization of Repeated DNA Sequences

There are no qualitative differences, in principle, between the basic genomic structure of plant and animal cells. Thus, the protein genes of plants, like those of animals, have a nitron-exon structure [14, 50, 93]. The plant genome has a much higher content of DNA repeats. This is particularly true of cereals. In the cereal genome, the repeats are some 75% of total DNA, in man about 20%, in the chick 20–30%, in the bull 25–30%, and in the mouse 40%. Cereals have a genome of much larger size (3.6–8.6 pg DNA per haploid genome) than man (3 pg). In the genome of larger size coding sequences and those accomplishing sequence-specific functions make up just 1% or so of the total DNA content [25]. With this in mind, the cereals are among the outstanding plants in the relative content of non-coding DNA.

2.1 CLASSIFICATION

It is premature to offer a classification of this great bulk of diverse non-coding DNAs. Here an attempt is made to consider them according to some of their general characteristics.

It has been aptly remarked that the repetitive component of DNA takes on

several guises [38], and the classification of DNA repeats according to their property of reiteration frequency, has gained acceptance since the 70s. Following Britten and Kohne [7] three large sequence classes according to renaturation kinetics are recognized. Their distinction is based on copy number of DNA sequences. Thus, the repeated sequences renaturation kinetics of plant DNAs have been used to distinguish unique sequences, middle-repetitive with 10–15^5 copy number and highly repetitive ones with still greater copy number.

In these experiments, DNA renaturation rate was very dependent on the reaction conditions, mainly on temperature and ionic strength of the solution [7]. The variation ranges for the Cot values (the product of DNA concentration and renaturation time) in assignment of the DNAs to kinetics classes were taken arbitrarily. Furthermore, the curve for the renaturation events had a discouragingly smooth course. For these and other reasons, the approach then used, although extremely useful, provided approximate estimates for the DNA kinetics classes [25].

The notion of satellite DNA still pervades in the literature. Satellite DNA is not distinguished by the copy number criterion. The DNA fractions have been isolated as satellites by the centrifugation of DNAs in buoyant density gradient. Satellite DNA (sDNA) represents 0–40% of the plant genome, and even closely related plant species within a genus can widely differ in the content of sDNA [5]. Detailed analysis has demonstrated that the sDNAs are, as a rule, simple, short, high DNA repeats tandemly organized in the genome [13, 26].

With the advent of molecular cloning technology, there appeared the possibility of dealing with individual DNA sequences. Heterogeneous mixtures and their confounding variable parameters were thus dismissed. There was a promise of treating sequence organization in the genome, copy number primary structure, the degree of homology or divergence in more precise terms. DNA sequences are now more often classified by their organization pattern in the genome as: (1) short, tandem arrays (the minority of repeats), and (2) repeats organized in a more complex than tandem manner. Some members of class 2 are interspersed with unique sequences and/or other different repeats (interspersion pattern).

Sequences of class 1 were found to be highly repetitious and, as judged from their kinetics characteristics, as well, they were referred to high repeats. Sequences of class 2 more frequently occur as middle-repetitive.

Clearly, the classifications of the repeats are different, because of the remarkable capacity of the same large classes of sequences to appear under different guises [38].

2.2 SEQUENCE INTERSPERSION PATTERNS IN CEREALS

There is a feature of genomic organization common to cereals to be noted [28, 29, 32, 65, 79]. It is close similarity in the relative content of certain DNA classes, kinetics complexity of the major bulk of sequences, interspersion pattern in the genome. The pertinent data in the literature are summarized in Table 1. Maize is an exception: approximately 40–50% of its genome consists of unique sequences [37].

Table 1: A characterization of some cereal genomes

Species	Genome size of the haploid (pg)	Sequences (% of total DNA content)		
		Unique	Repetitive	
			Total content	High (C_0t=0.01)
Weat *Triticum aestivum*	5.7	25	75	7–13
Rye *Secale cereale*	8.3	25–30	70–75	10–12
Barley *Hordeum vulgare*	5.5	30	70	10
Oats *Avena sativa*	4.4	30	70	not estimated

Evidence has been obtained for the interspersion of short repeats (mainly 200-400 bp long) with different unique sequences (with an average length of 700-1.000 bp) in a large portion of the cereal genome. The proportions of the genomes with this kind of organization have been estimated as 40-50% for oats, 50-60% for barley, 40-45% for hexaploid wheat, 30-35% for rye [29, 69, 70, 80]. Most of the genome of these cereal species is occupied by palindromes and tandemly arrayed repeats of two types: (1) consisting of very similar repeats, and (2) complex permutations of different repeats.

The genome organized with the interspersion pattern of relatively short unique sequences and short repeat units has been first described for *Xenopus laevis* [12], and now the term interspersion of *Xenopus-type* is of common usage. And the cereal genome is considered to have an organization of the *Xenopus-type*. However, no sooner a rule is established than exceptions are disclosed. Indeed, the millet genome stands aloof from other higher plants in its genome organization. Its genome is small, just 0.22 pg per haploid set [94], about 40 times smaller than that of rye. The millet genome is almost devoid of short repeats, and the unique sequences of the *Xenopus-type* are interspersed with repeats about 4.000-5.000 bp long [94]. Although small, the millet genome manages to harbour a percentage of unique and repetitive sequences that is almost as high as in other cereals. As to the maize genome, unique sequences interspersed with repeats stretch over a distance of 2000 bp [37].

2.3. DIFFERENCES IN THE CHARACTERISTIC PROPERTIES OF TWO REPEAT CLASSES

2.3.1. Chromosomal locations

The two large classes of sequences have other characteristic properties besides differences in copy number and organization of repetitive DNA in the genome. Predominant chromosomal location is one such characteristic property. The chromosomal location of short tandemly arrayed highly repetitive DNA has been thoroughly studied in cereals. The major sites for highly repeated DNA sequences have been identified in the telomeric regions in rye

[2, 44, 72] and in the precentromeric regions in barley chromosomes [72] by *in situ* hybridization. This location conforms with the distribution of heterochromatin in the corresponding chromosome regions. As to hexaploid wheat, large highly repetitive DNA blocks have been located on seven *B* genome chromosomes and also on two *A* genome chromosomes (chromosomes 4 and 7); it is of interest that these blocks were found to occur in the interstitial, telomeric and precentromeric regions [13, 32]. Longer exposure times of the autoradiographs have made possible to locate several minor sites for high repeats on the *A* and *D* genome chromosomes in wheat [13].

There is much less information concerning the chromosomal location of repeats of class 2. Their suggested dispersion throughout the genome is supported by the results of the *in situ* hybridization experiments [87]. Figure 1 clearly shows that the two classes of repeats differ in chromosomal locations. There are no predominant locations in the chromosome for the middle repetitive DNAs, as in the case of the highly repetitive ones concentrated in the precentromeric (barley) or telomeric (rye) regions.

2.3.2. The accumulation rate of changes in the primary structure

No consideration of the DNA repeats can be complete without an evolutionary framework. It seems reasonable to assume that some of the heterogeneous assortments of sequences with various properties have been spared by evolu-

Fig. 1: A comparison of the chromosomal location of the middle and high repetitive sequences based on *in situ* DNA hybridization:
A) ^{3}H-middle repetitive sequences of rye on the rye chromosomes.

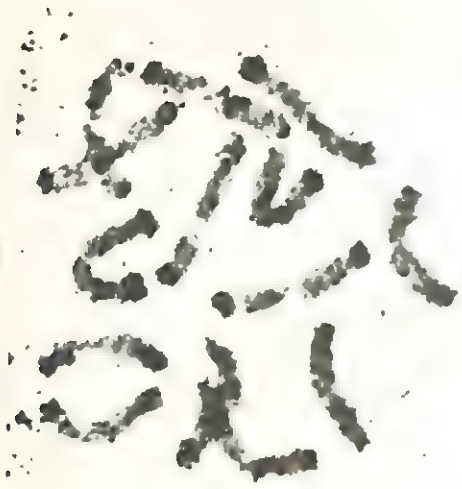


Fig. 1: B) ^{3}H-high repetitive sequences of rye on the rye chromosomes.

tion and, consequently, are common to, at least, some of the contemporaneous species, while others, as a result of great divergence of other events, have become species-specific. Meaningful estimates of sequence content in the cereal family have been obtained [28, 69, 70]. By hybridization of DNA sequences from wheat, rye, barley and oats, it has been demostrated that 16, 22, 28, and 58% of the respective DNAs are species-specific repeats arisen,

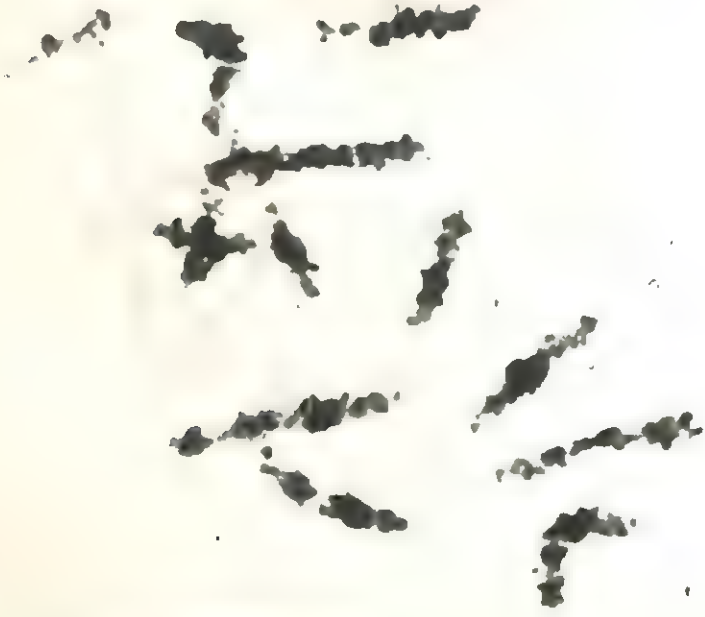


Fig. 1: C) ^{3}H-high repetitive sequences of barley on the barley chromosomes.

Table 2: Hybridization of ^{3}H-high and middle repeats with total DNAs from cereals immobilized on nitrocellulose filters (%)

Repeats	Total DNA			
	Wheat	Rye	Barley	Oats
^{3}H-high				
Wheat	100	80.8 + 3.3	62.4 + 4.9	24.8 + 1.6
Rye	54.7 + 4.1	100	60.4 + 4.3	-
Barley	75.1 + 4.2	75.0 + 3, 6	100	32.9 + 4.3
^{3}H-middle				
Wheat	100	80.4 + 4.5	71.9 + 3.7	35.2 + 2, 4
Rye	76.0 + 3.5	100	57.8 + 4.5	-
Barley	72.5 + 4.4	60.6 + 4.3	100	38.6 + 3.5

60% of the ^{3}H-DNA repeats hybridized with total DNA in the homologous hybridization. The level of homology hybridization was accepted as 100%. The values are means for 3–9 measurements. The confidence limits are at $P = 0.95$, Student's test.

probably, as a result of amplification of unique sequences after the species has diverged. Furthermore, the results obtained for homology between the sequences of the four cereals agree well with the generally accepted scheme of the phylogenetic relationships of cereals.

We have compared the extent to which fractions of high and middle repeats from cereal DNAs are heterogeneous [72]. As the data of Table 2 show, the classes have repeats in common, and species-specific ones too. The homology within each classes of repeats is the same as the one observed for the entire repetitive DNA by Flavell *et al.* [28]. There are some features of our intraspecies DNA/DNA hybridizations to be noted, however.

Thus, when ^{3}H-high wheat repeats were hybridized to total rye DNA (direct hybridization), the percentage of sequences common to both species was higher (80.8%) than when ^{3}H-high rye repeats were hybridized to wheat total DNA (54.7%, reverse hybridization). There was a consistent discrepancy between the homology percentages in all the direct and reverse hybridizations of the high repeats from all the cereals DNA studied. Under the hybridization conditions used, all the families of high repeats common to two species are expected to renaturate completely. With this expectation, two explanations were offered for the discrepancy between the direct and reverse hybridizations:

1) The two species presumably contain different percentages of shared sequences in the fractions of high repeats. These differences might have arisen because a shared sequence(s) has been amplified by different number of times after the divergence of the species.

2) Two plant genomes are presumably different in the percentage of species-specific families of high-renaturating DNA sequences.

Based on the homology percentages, the following conclusions were made:

a) The homology percentages were very close in the interspecies DNA/DNA hybridizations, using moderately repetitive sequences in the cross-combinations involving rye, wheat, barley, and oats.

b) The higher homology between the fractions of middle repeats, as compared with those of

the high ones, in hybridization of ^{3}H-wheat repeats to total barley and oats DNA, ^{3}H-rye repeats to total wheat DNA, as well as ^{3}H-barley repeats to total oats DNA. Thus, the results indicate that the middle-repetitive cereal DNA might have been spared by evolution. Consequently, it is the conserved portion of the genome.

2.3.3. Transcriptional activity and transposibility

The dispersed moderate repeats have been by far less studied in cereals than in the traditional genetic objects, for example, *Drosophila*, mouse and man. Thus, neither ubiquitous human *Alu*-family type, nor of rodent equivalent *Alu* type sequences have been, so far, found in plants. A distinguishing feature of the two repeat classes will now be considered, namely transcriptional activity and considerations will be limited to the experimental data obtained with the animal cell genome.

There is little information concerning the transcriptional activity of highly repetitive tandem sequences, which are, as a rule, located in the inert heterochromatin regions [83, 86]. In contrast, the short repeats interspersed with the unique sequences transcribe actively [43]. They are represented by heterogeneous nuclear RNAs (Hn RNA) and mRNA. We are not concerned here with sequences accomplishing known functions, those encoding rRNA, tRNA, histones, storage proteins occurring in tens and even hundreds of copies, in the cereal genome. The biological role of transcripts with repetitive sequences is unclear [43].

It has been found that the primary structure of the ubiquitous human *Alu* and mouse B1 sequences, as well as of many others, has the features of transposable elements. Pertinent data did not fail to come in abundance. Mobile elements are being discussed with reference to the structure and function of the genetic apparatus in eukaryotes. Transposable genetic elements at the DNA level, was a timely coined term [19]. We must not forget that the first discovery of transposibility was McClintock's [54] in maize. Whatever the level of its investigation or object may now be, it is her footsteps that are followed [36]. Because the basic idea of transposibility was spelled out for a plant crop, it seems appropriate to deal with it here in a separate section.

3. Mobile Genetic Elements (MGEs)

There is good reason for believing that the major portion of the eukaryotic genome is unstable; stating otherwise, it is able to transpose from one site of the chromosome to another under certain influences. The following estimates may give an idea of the implication of transposibility. To illustrate, 12% of the *Drosophila melanogaster* genome consists of moderately repetitive DNA [8], and about three-fourths of this component (with the exception of the tRNA, rRNA, 5S RNA genes and histones) are represented by genetic elements that are potentially mobile [38]. This would mean that the genome is tenanted by many elements able to move across it. There is little doubt that the size of the unstable portion of the genome varies in a wide range from species to species.

Can it be regarded as parasitic DNA? Is a role, all of their own, assigned to the mobile elements? What is the biological function, if any, inherent in the primary structure of their DNA? At this juncture these questions remain

open. What is obvious is that, integrating into specific genes and departing from a region of the genome, the MGE alter the expression at a particular loci and those neighbouring it. But what are the consequences of the integration and departure, the reminiscences, in the genome? The whole migration act may be just of the kind regulating the expression of the cell genetic apparatus. If so, it may be a passive act, engendering no far-reaching consequences. Before turning to the functional role of the MGEs, it would be worthwhile to describe at length this repetitive class of the plant genome.

Maize enjoys the most favourable status in the realm of transposibility, and features of its MGE will be considered. These are transposibility, mutability, phenotypic expression at the loci concerned, integration into instability sites.

3.1. TRANSPOSIBILITY

Maize MGEs transpose from one chromosome to another [56], from an unstable allele of a gene to the same locus of a homologous chromosome [35]. Some regularities in the transposition of the maize MGEs have been summarized [46].

(1) The greater the distance between the sites of transposition, the rarer their transposition occurrence; (2) the once transposed elements, after moving again, can return to the original locus, thereby generating a second mutation; and (3) the same element can transpose to distinct genetic loci. For this reason, there is no doubt that a plant containing a mobile element can dispatch it to any other gene [23]. This is in keeping with the observation that transposition is not very much site-specific.

In the case of precise excision of elements, the genes concerned revert to their original state, and the stable wild phenotype is restored. If the excision is imprecise, the locus remains altered, but becomes stable. The transposable elements either make themselves at home in their new residence or seek yet another, i.e. the elements behave in a stable or unstable manner. It is unclear what determines this behaviour of the MGEs: differences in the new integration sites or altered structure of the MGEs *per se* [23]. The local structure of the chromosome strongly affects the transposition behaviour of the elements, like any other event of genetic recombination [46].

Transposition frequency is related to many factors including [46].

1) The distance between the MGEs and the heterochromatin. The proximity of heterochromatin has a stabilizing effect on the MGEs.

2) The direction of the cross.

3) The properties of the MGEs. Changes in these properties are primarily manifest in changes in mutability at the loci sites, into which the MGE integrates or in shifts in the developmental timing of the mutation. These changes in MGEs properties are, as a rule, reversible, and they are known as the state.

4) Genetic background.

Transposition is more often than not associated with marked chromosome rearrangements. The DS element is peculiar in this respect. It is non-autonomous (incapable of autonomous transposition) and causes chromo-

some damage. The occasional consequences of its transposition are large chromosome rearrangements, of which one is a break at the original locus, and the other at the new site of the transposed DS copy. The excision transposition mechanism of maize MGEs is similar to that of the mobile elements of *Drosophila*, but different from that of bacteria.

3.2. MUTABILITY

This characteristic of loci containing the MGEs varies greatly, even for the alleles of a gene. Mutability can attain about 10% for some loci and 0.1-1.0% for others [56, 60]. The explanations offered for these variations are that, either the alleles may differ in the location sites of MGEs, or the differences may be due to structural changes in the elements [60].

Mutability of the alleles containing the non-autonomous MGEs appears to be much more related to the dosage of the autonomous MGE in the other sites of the genome. This appears plausible, because of their capacity for making the non-autonomous members move elsewhere [30, 55]. However, the MGEs differ considerably from family to family in dosage effect [23]. Thus, mutability of the transposition-prone alleles increases (the family of the *Dt* elements), decreases (the *Ac* elements) or remains unchanged (the *Spm* family) with increasing number of elements cohabiting in the genome [34, 35, 54].

Thus factors determining the level of mutability of the unstable alleles are of various kinds: original location of the MGEs; their properties and number, temperature conditions, the stage in the life cycle of the organism [46].

3.3 VARIATIONS IN PHENOTYPIC PATTERNS

Unstable loci can give various patterns of phenotypic changes: from mutant to wild or close-to-wild (stable or unstable) type [55]. There are instances when mutations give rise to a series of alleles differing quantitatively in the expression of the wild trait. The majority of unstable genes with integrated MGEs express themselves as amorphic or hypomorphic alleles with decreased or no gene product [22, 74, 75]. From these facts it is obvious that the MGEs can decrease the activity of genes, but not to the degree of its abolition. In case of precise excision of the MGEs, the synthesis of the gene product is restored. However, many mutable loci can generate intermediate series of alleles controlling different levels of the elevated synthesis of the needed gene product [61, 67].

Thus, most of the mutations at unstable loci give rise to unstable loci, causing quantitative changes in the synthesis of the needed product. However, the changes can be qualitative. The consequences of the insertion of the *Ds* element into the *Bz* locus are also of quantitative kind [17, 18]. It has been found that protein is missing in some of the mutants during all the stages of endosperm development. The timing of the synthesis of glucosyl transferanse sharply deviated from the normal, as well as its thermolability characteristics, in one case under the effect of the allele *bz-m4*.

Studies of the stable phenotypic revertants derived from the mutant *bz-m2* yielded interesting results. Thus, the reverse mutations produced by the *Ac-Ds* system proved to be very heterogeneous [17, 18]. They were not true revertants to the wild type in their majority, because the structure of the loci

was not amenable to complete restoration by excision of the *Ds*. Imprecision of excision of the element may be one of the causes why the protein products are differently changed during the partial reversions to the stable loci.

The subtlety of the response of genes to inserted elements has been providing the selective forces of the natural selection with renewed material. This rich subtlety can be, possibly, taken advantage of in the breeding of crop plants, mainly maize [46]. The MGEs systems have been, with very good reason, called one of the sources of plant heterogeneity [56].

3.4. PRIMARY STRUCTURE

The maize MGEs have the ability to transpose autonomously (autonomous elements, regulators), or under the influence of autonomous elements present elsewhere in the genome (receptors). A regulator and receptor are, as a rule, incorporated into a MGE system, which may consist of a set of different regulator and receptor elements.

The maize transposable elements stirred interest, because of their non-Mendelian behaviour and thought-provoking molecular basis [19]. The maize Activator (*Ac*)-Dissociation (*Ds*) system seems to be the one most studied in molecular terms. Here again, we owe our debt of gratitude to McClintock for isolating the mutants, which have helped to unravel the primary structure of the MGEs as a whole. The *Ac* and *Ds* elements produce mutations at the *shrunken* locus encoding endosperm sucrosesynthase, at the *waxy* locus encoding glucosyltransferase and also at other distinct loci. The insertion of these elements has slight effects on the viability of the mutants.

A brief characterization of the primary structure of the *Ac-Ds* system follows. Two *Ac* elements independently isolated from two different *waxy* mutants have a length of 4563 bp [58, 62]. The *Ds* differs from the *Ac* elements in a central deletion, which may abolish the ability for autonomous transposition. The *Ds* elements have been isolated from the *ux*, *sh*, and *Adh 1* mutants, and deletion lengths vary from 194 bp to 4.531 bp [20]. The *Ac* and *Ds* elements have inverted sequences at their ends, and they generate duplications at their insertion site. The termini of *Ds* consist of 11 nucleotides TAGGGATGAAA; *Ac* has the same primary structure as *Ds* with the difference that the outermost nucleotide *A* is replaced by *C* in *Ac*. A salient feature of these two elements is the presence of many direct and several inverted repeats of different lengths. Doring *et al.* [20] consider that the organization of the repeats in the *Ds* and *Ac* is not random, but its significance is unclear. The *Ds* can be double, inserted in inverted orientation of a copy of itself [20].

The *Ac* element is inserted closer to the 5′-terminal locus in the *wx-7*, and it is inserted in an exon in the *wx-9* mutant locus. Open reading frames have been located for the *Ac* element; although *Ac* contains sequences resembling the signal sequences found in eukaryotic genes, there is no certainty that transcription (or translation) occurs in it [20].

To reiterate, the phenotype can revert to normal, completely or partially, after removal of the MGE from the locus into which it has inserted. Sequence analysis of revertants for the *Adh 1*, *waxy* and *sh* genes has demonstrated that the integrated *Ds* copy present in the duplicated sequences of target DNA

undergoes a partial asymmetrical deletion [19]. In the case of the excision of other MGEs, the *Tam 3* element of *Antirrhinum majus*, for example, uncleotides appear *de novo* in target DNA [82].

The MGEs have been discovered and investigated in other plant species, besides maize: *Glycine max* (the *Tgm* element); *Antirrhinum majus* (the *Tam 1, Tam 2, Tam 2* elements). These species are taxonomically, i.e., evolutionarily quite distant, and they belong to different families. They are, nevertheless remarkably similar in their structural organization [89]. Thus, the elements *Spm* (maize), *Tgm 1 and Tam 1* have almost identical terminal inverted repeats; *Spm* contains hexanucleotide ACACTC, and *Tam 1* contains septanucleotide ACATCGG within their structure. All these three elements produce in the target gene a duplication involving three nucleotides. And, what is more, there are structural features common to the MGEs of animal and plant cells. Homologous terminal sequences have been observed in the maize MGE *Cin 1* and in the *Drosophila copia* elements [77]. The *Drosophila FB*—transposon contains a repeated structure, a decanucleotide with the same first five nucleotides as in the maize *Ds* element [20]. The evidence for this structural parallelism refutes randomness. It rather argues for evolutionary conservation of some of the MGEs regions. The horizontal transmission of the elements with the same termini from one organism to another of different taxonomic rank is a possibility to be considered seriously.

3.5. OCCURRENCE OF THE *DS*-LIKE SEQUENCES IN THE CEREAL GENOME

It was not at all an exaggeration to say that the transposable elements were just awaiting to be discovered in other objects besides bacteria, yeast, maize and *Drosophila* [84]. The evidence for the evolutionary conservatism of the MGEs (previous section) prompted us to determine whether maize *Ds*-like sequences can occur in the genomes of other cereals. The results related to the experiments we carried out, have been presented in detail elsewhere [88]. The *Ds* element from maize DNA has been used in these experiments. This *Ds* element spans 2.7 kb in the *shrunken* locus isolated from the mutant maize *sh m-5933* and excised from the *Bam* HI sites of the locus [31].

Assurance that the *Ds*-like sequences are, indeed, present in the cereal genome was provided by dot-hybridization [45]. The filters were washed under gentle and stringent conditions. The temperature at which the filters are washed after hybridization is a known criterion of stringency. When the homology between the prove and the filter-bound DNA is poor, the duplexes can dissociate under stringent conditions. The *Ds*-like sequences were found to be present in the genomic DNAs of rye, wheat and barley. The pertinent data are given in Table 3. The number of the *Ds*-like sequences in the genomes of rye, wheat and barley varies from 100 to 300, which is an order of magnitude less than that in the maize genome. It should be noted that the differences in the variation range between *Triticum, Secale* and *Hordeum* are small.

While not affecting the self-hybridization of the *Ds*-element, the more stringent conditions produce a more than two-fold decrease in copy number in the cereal genomes (Table 3). Thus, the cereal genome contains a fairly

Tabe 3: The copy number and the T_m of the *Ds*-like sequences in the cereal genomes

Species	Genome	Copy number*			T_m (C°)
		Gentle washing conditions, 20°C	Stringent washing conditions, 55°C	Loss %	
Tr.monoccocum	A	55	26	47	
Tr.diccocum	AB	119	57	48	
Tr.timopheevi	AG	83	43	52	
Tr.aestivum	ABD	218	94	43	46
S.cereale		337	155	46	45
H.vulgare "Winer"		160	70	48	45.5
H.vulgare "Nepolegaiushchii"		185	85	46	
H.vulgare "Galina"		178	93	52	
H.vulgare "Sophia"		149	61	41	
Zea mays		1950	—	—	52
Ds sh-m5933		—	—	10	54

* Copy number was determined as mean values for three to five experiments.

high number of *Ds*-like sequences showing different degree of homology to the *Ds sh m-5933*.

The results raised the question of the degree of the homology between the primary structure of the various cereal *Ds*-like sequences and of the maize *Ds* element. An attempt was made to answer this question by comparing the melting point (T_m) for the DNA duplexes formed as a result of the hybridization of the *Ds* element with the cereal DNAs. As the data of Fig. 2 and Table 3 show, the Tm of maize DNA is lower by 2°C than that of the selfhybridized *Ds* element. A decrease of 1°C in the T_m is produced by a substitution of 1.0–1.2% of nucleotides [1, 92] and, hence, there would be 2.0–2.5% of substituted nucleotides in the *Ds*-like sequences of maize genomic DNA. However, wheat, rye and barley DNAs do not differ significantly in the T_ms, which are much lower than the one for the *Ds* element. The standard deviation found by Marmur and Doty [53] for repeated determinations of the T_m curves is +0.4°C. Those for the *Ds*-like duplexes in *Triticum*, *Secale* and *Hordeum* rise more sharply than that for the *Ds* element at low temperatures (Fig. 2). This shape of the curves may be the consequences of the high heterogeneity of the *Ds*-like sequences and of large variations in their lengths.

Is a reduction of 8–9°C of the T_m values for the *Ds*-like sequences significent compared to those observed for other kinds of repeats? The comparative data concerning the primary structure of the spacer regions of wheat rDNA are of relevance: when a untranscribed wheat rDNA spacer region 130 bp long was hybridized with rye DNA, the T_m for the formed duplexes decreased by 17.5°C; in the case of the hybridization of a 750 bp spacer from another region, the T_m was slightly, if at all, different [1]. Thus, the differences between the cereal T_ms, as judged from our results, and the T_m maize *Ds sh m-5933* are within the range of the previously established interspecific differences [1]. The data suggest that the genome of wheat, rye and barley contains a set of

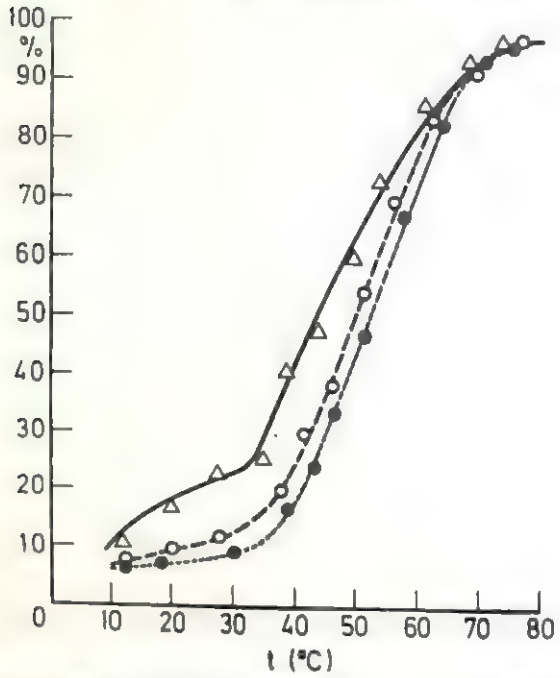


Fig. 2: The melting curves of duplexes formed between *Ds sh-m5933* and DNAs from
..... *Ds sh-m 5933*
— . — . — . — *Zea mays*
———— — *Tr. aestivum*

Ds-like sequences, heterogenous with respect to the length and homology to the *Ds* element of the maize locus *sh m-5933*. The data further suggest that the *Ds* elements may strongly influence gene expression in the presence of the *Ac*-like element, as well as of other sequences able to transpose autonomously.

Thus support was obtained for the MGEs being integrative components of the eukaryote genome and their repeats. It also appears obvious that other elements, besides the *Ds*-like, are residents of the genome of the cereal species we studied. The possibility of the MGEs having a future in the past, figuratively speaking, has been envisaged [46]. It might be imagined that genetic factors, pre-existent to the MGEs, would be inactive and, consequently, left behind the scene; activating influences (some of which will be discussed in the next section) of some kind would throw some light on the hidden transposable factors. The nature of the precursors of the MGEs is speculative. They may be ordinary genes, although with important assignments, inconsequential or even stray sequences. McClintock's [55] predictions concerning the origin of the MGEs from heterochromatin, which is saturated with repeated sequences, should be recalled in the present context. The concentration of the repeats in the MGE termini supports and extends these predictions.

4. Plasticity—An Important Feature of the Plant Repetitive DNA

Having considered some of the features of repetitive DNAs, which are not involved in the encoding of protein molecules (histones, storage proteins and

others) and RNA (rRNA, tRNA), it would be expedient to turn to their functional role in the plant cell genome. Following is a brief review of what is known about it. The mechanisms underlying changes in DNA structure will also be discussed in the context of the functional role of the repeats.

4.1. MECHANISMS UNDERLYING CHANGES IN DNA STRUCTURE

Flavell [25] has assumed that an amplification mechanism is predominantly responsible for the production of repeated DNAs, especially highly reiterated ones. As noted in section 2.3.2, the high and middle repeats contain sequence families. These sequences are members of the same family, provided that they are homologous to the degree, allowing the formation of stable two-helical structures at appropriate conditions. Flavell [25] has also assumed that repeats, members of the same family, have arisen from a unique sequence, as a result of an amplification act(s). Amplification or selective replication has been first described for the genes encoding *Xenopus laevis* rRNA. An example of the amplification of the gene for dihydropholate reductase, an enzyme whose activity is blocked by the toxic agent methotrexate, came from the studies of Schimke *et al.* [73]. Other examples can be adduced. Genes needed to programme the normal stages of cell development can be amplified (the chorion proteins of *Drosophila*). The amplified gene copies, as a rule, exist as extrachromosomal cell elements. They are lost in subsequent cell divisions; occasionally, however, they are integrated into the chromosome genome to become its fullfledged member. An illustrative case is that of the genes for dihydropholate reductase in cell murine lines resistant to methotrexate [73].

These examples concern amplification that occurs when the cell acutely needs the product(s) encoded by the amplified gene(s). There is also a spontaneous level of gene amplification independent of selective pressure [90]. Extrachromosomal DNA has been described in pea plants (*Pisum sativum*) grown under conditions excluding obvious selective pressure [48, 85]. The reports dealing with spontaneous gene amplification are scant, and the whole phenomenon including its causes has the lure of nuclear implications.

A feature of high repeats is to be reemphasized, namely its organization in long tandem arrays. The same sequence can be tandemly arrayed in all the chromosomes, like the high repeats of rye DNA [2, 44]. This organization may be an indication of the ability of sequences to transpose from one chromosome region to another, and to the same chromosome site, the telomeric in rye, for instance. Why is the location site for a transposition the same? Does this make any difference to the functioning cell genome? Or, perhaps, a site is preferred because a sequence cannot move to other than it does and continue to exist?

It has been demonstrated that highly repetitive DNAs located in the precentromeric and telomeric regions are of importance in establishing the chromosome coorientation in the interphase cell nucleus and also in providing regular mitosis and meiosis. Based on comparative study of the location of four families of high repeats in rye variety King II and additional lines of *Tr.aestivum*, Jones and Flavell [44] have concluded that the families are

organized independently of each other and in a strictly ordered pattern in the telomeres. This pattern facilitates the association of the telomeres of nonhomologous chromosomes in the interphase nuclei, when all the chromosomes, according to the models of Bennett [4] and Shchapova [76], are arranged linearly at the cell pole opposite to the one where the centromeres lie. The association between the telomeres may be of importance in the replication of the chromosome ends [39].

The view has been held that the amplification mechanism is associated with translocation [26]. Without going into any detail of this mechanism, it appears that the model of rolling ring and rapid integration has gained wide acceptance [40], and changes in copy number in the tandem repeats have been accounted for by unequal crossing over [78].

Amplification can bring about an increase in the total DNA content in the cell and in small chromosome regions, thereby disproportionately increasing the size of these regions. There is a good body of evidence indicating that increase in the amounts of DNA in the cell with concomitant increase in its size may decelerate mitosis and meiosis [4, 51, 68]. High DNA concentration in a chromosome segment may have an undesirable outcome. It is pertinent to recall that Lima-de-Faria [49] has opposed the concepts of simplicity and economy to those of complexity and uniqueness, and chaos in chromosome organization to optimal territory for each gene within the chromosome. As a corollary, it would follow that there may be a process counteracting amplification, namely one deleting DNA. This implies a secondary phenomenon creating a deviating situation in the vein of Lima-de-Faria [49]. The idea of an optimal territory occupied by each gene within the centromere-telomere field of a chromosome [49] is not at variance with that of abundance of mobile elements in the eukaryotic genome. As a matter of fact, transposition frequency is indirectly related to the distance at which the MGE is transposed, and the transposition of elements from one chromosome to another distinct one is very rare.

4.2. EVOLUTIONARY CHANGES IN THE GENOME

A certain part of the genome displays marked variability when compared to its other part. The unique sequences, at least those in the regions concerned with the encoding of metabolic enzymes, have a structure with stability maintained by natural selection. The repeated sequences are most susceptible to change resulting from amplification, translocation, deletion, mutation and the other tools of evolution, tinkering novel genomic configurations. Sequences in new environments may, quite conceivably, repattern sequences and, when associated with amplification, give rise to new ones within whose confines repeat families change their positions. But we well know that in the face of change, there persists a staunch loyalty to the old. There is an undeniable looking alike in genetic makeup that cannot be rid of completely, no matter how annihilating the evolutionary forces may be. *Plus ca change, plus c'est la meme chose*? The term homology suggests itself from Vavilov's sense in his law of homologous variations to that now implied in comparisons of the sequences of plant cell DNAs.

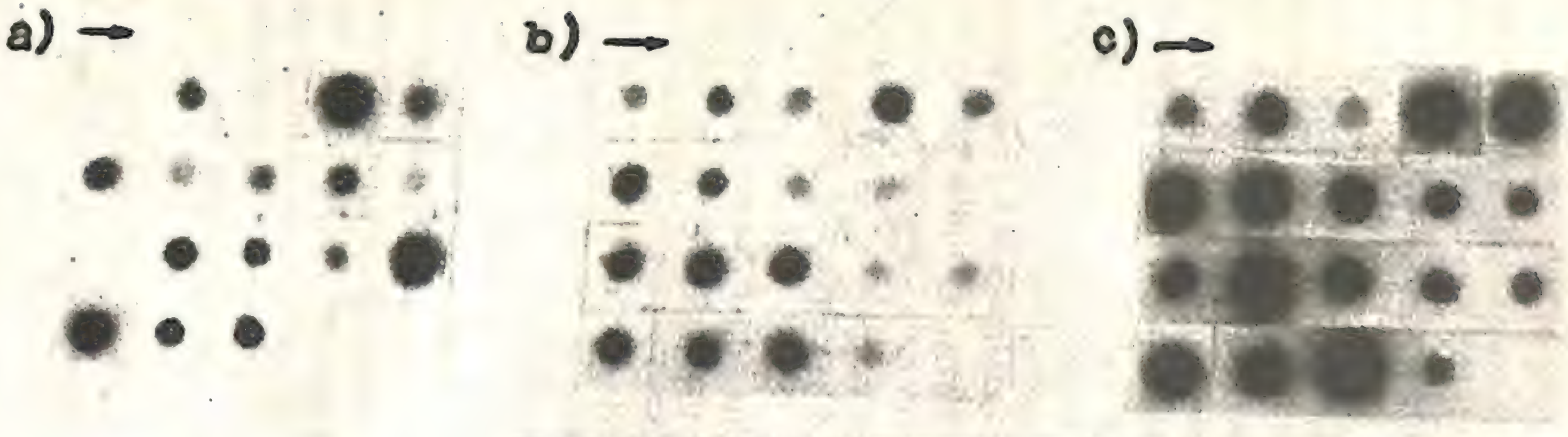
The degree of homology between two kinetics repeat classes of four cereal species has been characterized in section 2.3.2. Based on these characteristics, the class of high repeats constitutes the most labile part of the genome. This is in agreement with the conclusion made by Cullis from crosses between high DNA and low DNA genotrophs of flax [90].

The *de novo* arising sequences are species-specific with respect to appearance frequency and proportion to total DNA content. A case in point is the comparison of the genomes of the closely related *Aegilops squarrosa, Aegilops speltoides* and *Triticum monococcum* [27]. This comparison revealed that almost all the repeated sequence families present in *Ae. squarrosa* DNA also occur in that of *Ae.speltoides* and *Tr.aestivum* DNA, and only a small proportion (0.5% of total DNA) of these families do not. A much greater percentage of the *Ae. speltoides* genome (2–3% of total DNA) was found to be composed of repeated families absent from the *Ae. squarrosa* and *Tr. aestivum* genomes.

As judged by our results (Table 2, section 2.3.2), the homology percentage of the highly repetitive sequences of barley DNA with the DNA from rye and wheat is high (75%). It exceeds by far that between the high repeats of wheat and rye DNAs (62% and 60%, respectively). This discrepancy in homology percentage may be taken to mean that, after divergence from a common ancestor, the genomes of wheat and rye evolved faster than the barley genome at the expense of the emerged species-specific sequences and their amplification.

It appeared of interest to analyse the high repeats of barley DNA [71]. For this purpose, 19 clones were chosen from a cloned library. The choice was based on the strongest signal in the hybridization experiments with barley total DNA. These clones were a set of sequences inserted into the plasmid pBR 327 at the *Pst*-site. The sequences varied in length from 80 to 600 bp and in the degree of homology. Their presence in the rye and wheat genomes was ascertained by dot-hybridization with ^{32}P-total DNA of these two species. The results presented in Fig. 3 show that the sequences in pHv 7263, pHv 7308, and pHv 7223 belong only to the barley genome, i.e., they are species-specific, pHv 7191, pHv 7241 belong to the rye and barley genome, and the remaining plasmids are common to the three genomes. The sequences from the three different genomic DNAs gave different hybridization patterns, thereby indicating that their repetitivity is different. Repetitivity was higher for pHv 7161, pHv 7179, pHv 7189, pHv 7191, pHv 7245, pHv 7290, and pHv 7302 in the barley genome.

Analysis of sequence organization in the barley genome by blot-hybridization allowed to reduce the number of sequences with individual characteristics. The hybridization patterns and, hence, the genome organization of the most common pHv 7161 and species-specific pHv 7308 are compared in Fig. 4. The comparisons demonstrated distinct differences between the hybridization patterns yielded by the two sequences. The scarcity of species-specific sequences among the barley high repeats was somewhat contrary to expectation, when taking into account that, of the four repeated families constituting the bulk of telomeric heterochromatin, three are species-specific [2]. Other



pHv7036, pHv7053, pHv7129, pHv7161, pHv7179,
pHv7189, pHv7191, pHv7197, pHv7220, pHv7223,
pHv7241, pHv7245, pHv7247, pHv7263, pHv7283,
pHv7290, pHv7293, pHv7302, pHv7308.

Fig. 3: Dot-hybridization of ^{32}P-total DNA from wheat(a), rye (b), barley (c) with the recombinant plasmids

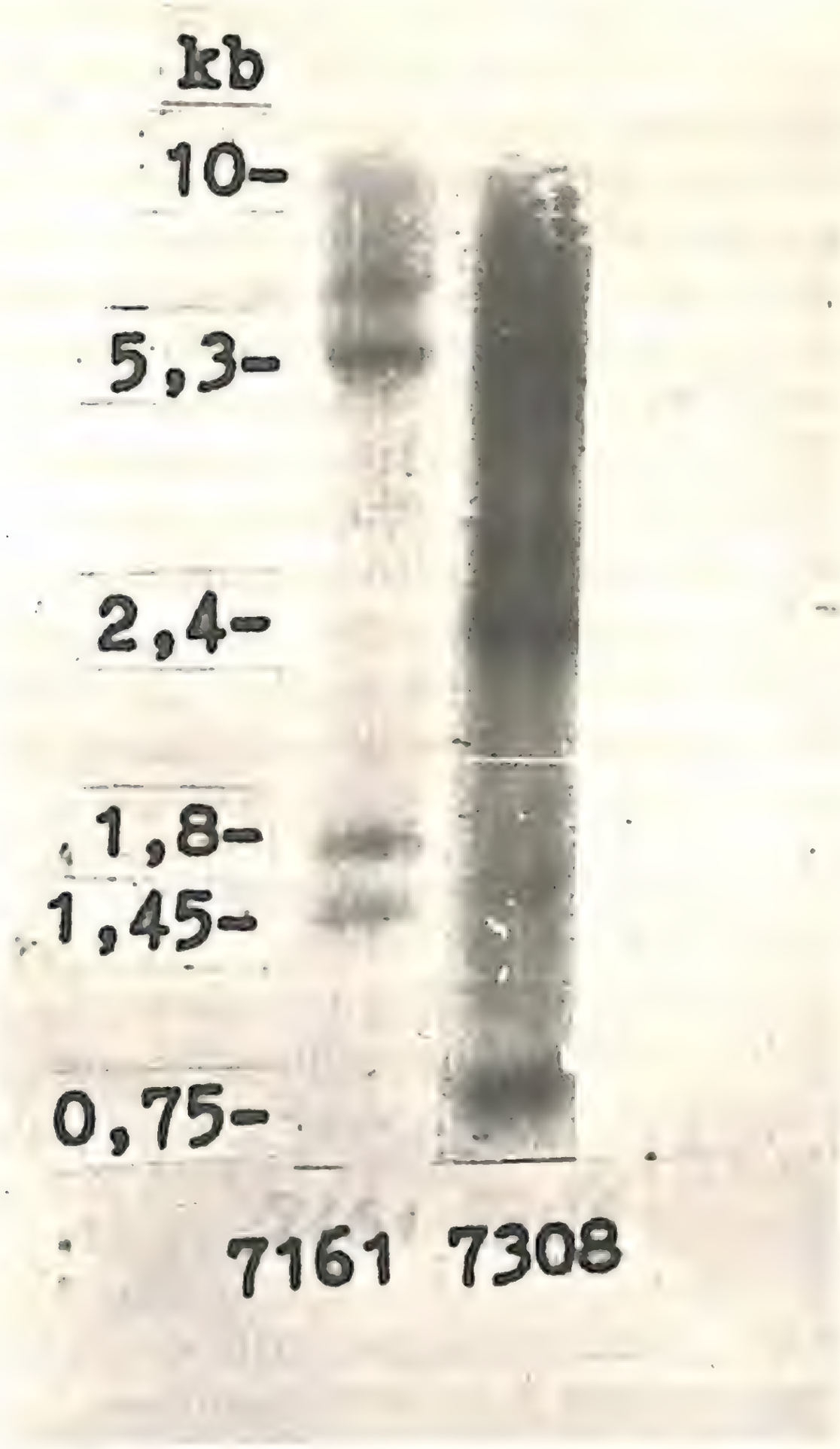


Fig. 4: A comparison of the genome organization of the high repeats of barley DNA common to the cereal genomes (pHv 7161) and species-specific to the barley genome (pHv 7308) by blot-hybridization.

species specific sequences may be present in the barley high repeats. These may have eluded our detection for many reasons, small size may have been one of them.

4.3. RAPID GENOMIC CHANGES

Continuous production of new sequences and reduction in the copy number of old ones increase, undoubtedly, genomic diversity in the plant, a circumstance of significance in the evolutionary scale. There is another aspect of the problem not to be overlooked, namely, the importance of genomic renewal as expressed by the lifespan of a single plant generation and its progeny. In such context, the term genomic plasticity would be appropriate. Genomic plasticity embraces all the genomic changes that take place during the lifespan of a single plant generation and that are of vital importance.

Genomic plasticity in the plant has to be considered with particular reference to a basic distinguishing feature of the plant and animal kingdom. Being mobile, animals can be in quest of better habitats, niches, if environmental conditions become harsh; being sessile, plants are permanently fixed

and cannot do so, and, hence, they are bound to rely on internal sources of variability. The degree of the plasticity of the plant genome is higher than that of the animal genome. As to the size of the plant genome and to the rate of its change, it has been observed that genome size is almost the same in the majority of mammals, in spite of large differences in the size and number of chromosomes. In angiosperms, genome size varies thousand-fold from less than 0.1 pg (*Arabidopsis thaliana*) to 100 pg (polyploids) [90]. This deserves attention when recalling that the first appearance of mammals on earth dates roughly 150 millions of years before that of angiosperms.

Genomic plasticity is specialized for reserve adaptation of the plant cell. A model treating the plant cell genome under stressing conditions will serve as an illustration. It is irrelevant whether stress is produced by fluctuating environment or modified genotypic background. What matters is that the plant cell genome is stressed to the point of taking recourse to its internal defense strategy.

4.3.1. Stress under altered conditions of growth

The accumulation of inherited changes in the genome of lines of flax provides a good case of stress resulting from altered conditions of growth (see review Cullis [9]). The stable flax lines differed from one another and from the original variety *Stormont Cirrus* in weight, plant height, content of nuclear DNA, the number of 25 S and 18 S rRNA, 5 S RNA genes and also in a large set of cloned sequences. The lines were grown under different regimes including modified concentration of nutrients, light intensity, temperature, etc. The variations in the copy number of repeats in the different flax genotrophs were found to be in the same range as in contemporaneous flax and its presumptive ancestral species. This suggested parallelism between mechanisms producing genomic change during a single generation and in a broad evolutionary scale [90].

4.3.2. Stress under conditions of tissue culture

In vivo transfer of plant tissue from natural to artificial conditions of growth in a cultural medium is stressful. This is manifest in genotypic and phenotypic variations. Changes at the level of the genome can concern the whole chromosome (chromosome loss), its extensive regions, even DNA sequences. Cullis has compared the genomes of leaf and callus tissues of flax using a number of cloned highly repetitive sequences [90]. In hybridization experiments, a set of cloned DNAs representing the great majority of the high repeats of the flax genome was used as a probe of the magnitude of variations for repetitive sequences between the genotrophs. Some lines showed no variations, while others did, and for most of the DNA probes. Clearly, genomic change is one of the ways cultured plant cells respond to the stress of tissue culture. The magnitude of variations for DNA and repeat content has been compared in the cultured and intact plant cells of *Rauwolfia serpentina* [81]. The maintenance of *Rauwolfia* cells as calluses produced an increase in ploidy level with associated multiplication of repeat copy number, and decrease in the degree of homology, at least in the part of the genome containing repeat units.

4.3.3. Stress of remote hybridization

Possibly, the strongest suggestion for remote hybridization being a form of stress for the hybrid plant genome has been made by McClintock [57]. There arises, beyond any doubt, a new state when two different genomes combine in a new one. Whatever the state may be called, disequilibrium, incompatibility, it all amounts to stress. But how can distant species and genes, when crossed, repattern and reprogramme themselves to cope with the alien genome? The point is that they do cope with the help of all the known reorganization devices (amplification, translocation, deletion, etc.) and, perhaps, unknown ones. The deviation, often marked, of total DNA content, or genome size from the expected parental, suggests that coping is achieved through genomic reorganization. An illustrative fact is borne out of the determination made by Price *et al.* [63] of the inheritance of the variation in 2C DNA values in interspecific hybrids of *Microseris* ssp. In *M. douglasii* × *M. bigloveii* crosses, the values for 2C DNA variation in F_1 hybrid progeny were within the range of the extreme parental values. The F_2 hybrid progeny were obtained from the F_1 plants and each F_2 hybrid plant had a 2C DNA value corresponding to that of the F_1 parent. The F_1 plants were not identical with respect to the 2C DNA value, because it showed no segregation for it. However, we must not forget to add that there are known instances when 2C DNA content is intermediate with respect to the parental one *e.g.* the crosses between *Lolium* species [42].

The most extensive analysis of the copy number of cloned sequences was based on its inheritance pattern in the F_1 generation of several inbred maize lines [90]: the copy number of repeats varied from two to five folds in eight of the lines, one was exceptional in showing no variations. The sequences represented different classes: the rDNA genes, sequences tandemly arrayed and dispersed throughout the genome. Some of the F_1 offspring from each cross were identical with respect to the presence of a sequence, but the majority displayed deviations from the expected parental average. The principles for the inheritance pattern of copy number formulated by Walbot and Cullis [90] may be rendered as: (1) change in copy number in a sequence is unidirectional, for example, the copy number of rDNA tends to reduce; (2) the magnitude of change is not related to the representativeness of a sequence in the genome, nor to the difference in copy number between the parents; (3) change in the copy number of a sequence is an independent event. The main inference of this study is that it is of no matter how the sequences are organized in the genome (tandemly or scattered) for a change to occur the copy number. The conclusion which stands out as one of the chief results of this work is that genomic changes are programmed by features of chromosomal organization.

5. Conclusion

When giving the name of Genetics to a still nameless science in 1906, Bateson has remarked (cited from *Scientific papers of W.Bateson*, 1928): "When formally we looked at series of plants produced by hybridization, we per-

ceived little but *bewildering complexity*. We knew well enough that behind that complexity *order and system* were concealed ... 'These words are called again to attention explicitly or implicitly when referring to the *bewildering complex* eukaryotic genome [26]. From hybridization in the field to DNA hybridization, there remains bewildering complexity resolved by *order and system*. Current conceivable ways of order installation would include, among others, regulative coordination programmed in the genome for the foreign integrator gene [6], the regulatory role of the transcripts with middle repeats [11] or programmed promotion of the amplification of some sequences and elimination of others [90]. Thus, it would appear that the term selfish DNA, was not so much suggested as a reflection of things, but as a semantic convenience. Respecting facts, the term will not do good service. Must the non-coding DNA be egoistic?

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3

Human and Animal Hepatocyte Xenobiotic Metabolism

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1. Introduction

The understanding of genetic factors that determine human metabolism of xenobiotics has increased greatly in the past few years. The uses of human cells or tissues in the study of carcinogen activation and deactivation have followed several lines of reasoning as outlined by Harris [26]: (1) environmental xenobiotics in many cases must be metabolized to a mutagenic or carcinogenic form, (2) the ratio of activation/deactivation may differ from individual to individual and may confer a higher or lower sensitivity to that particular chemical, and (3) comparisons of human to animal xenobiotic metabolism can be useful when extrapolating rodent carcinogenesis data to humans.

The increasing studies of human metabolism have focused attention on the contribution that such human data may have in a risk assessment process. The ultimate goal of this process is to protect man from exposure to toxic or carcinogenic chemicals. However, most data on toxic or carcinogenic effects of environmental chemicals are obtained in nonhuman species and must be extrapolated to humans. The information presented herein will provide examples showing that human metabolism can differ from that of other species, but that the difference is primarily quantitative. In addition, interindividual differences in metabolic capability within the human population will be illustrated and the effects of these differences on the extrapolation process will be discussed.

1.1 METABOLISM AND THE CARCINOGENIC PROCESS

The exposure of humans to chemical carcinogens may occur via dermal contact, ingestion, inhalation or by a combination of these routes. Although some carcinogens are direct-acting agents, the majority of environmental carcinogens to which humans are exposed require metabolic activation in order to initiate the carcinogenic process [49]. Because of the importance of carcinogen biotransformation in the formation of reactive as well as detoxified products, metabolic studies with known or suspected carcinogens have been a focus of intensive research. Certain model chemicals, such as benzo(a)pyrene, 2-acetylaminofluorene, and dimethylnitrosamine have probably received the greatest attention and have provided insight for studies with numerous other chemicals. Also because of the liver's broad metabolic capability, this tissue and various preparations made from it have been extensively used to study xenobiotic biotransformation. Furthermore, from a physiological standpoint, the liver is the primary organ for blood flow from the portal and systemic circulation and xenobiotics can reach the liver regardless of the route of exposure. Some carcinogens upon activation in the liver may exert their tumourigenic properties in the liver itself [67] or potentially reactive metabolites or conjugated products may induce their carcinogenic activity in other organs. Metabolism of xenobiotics occurs in other organs also; but relative to liver, they have received less attention for study.

The enzyme systems in the liver for carcinogen biotransformation consist of Phase I and Phase II reactions [29]. Phase I reactions introduce or expose functional groups via oxidative, hydrolytic or reductive pathways. Phase II reactions result in the formation of polar products due to the conjugation of

glucuronide, sulphate, glutathione or an amino-acid moiety to a Phase I product [14]. Depending on the nature and dose of the chemical, varying amounts of Phase I and/or Phase II products are released from the liver. Because of the importance of the metabolism process, it is essential that an accurate knowledge of a chemical's metabolism be acquired to understand and/or predict the possible toxic, tumourigenic or mutagenic impact.

1.2 CYTOCHROME P-450 ISOZYMES IN HUMAN AND RAT LIVER

The cytochrome P-450 dependent mono-oxygenases are the major enzymatic systems that catalyse the Phase I metabolism of many carcinogens [43]. This enzyme system is present as a membrane bound component of the endoplasmic reticulum [29]. The major component of this system consists of cytochrome P-450 hemoproteins, which bind the chemical and catalyse the reaction resulting in the transfer of oxygen to the compound. The other major components are two enzymes, NADPH cytochrome P-450 reductase and NADH cytochrome B-5 reductase, which function in an electron transport capacity, shuttling electrons to the P-450 hemoproteins [29]. The P-450 system catalyses many reactions such as N- and O-dealkylation, side chain oxidation, aromatic hydroxylation, sulphoxidation, epoxidation and N-hydroxylation [12, 29, 51].

Many functionally related multiple forms of cytochrome P-450 have been isolated over the last few years, especially from rat, rabbit, and human tissues [7, 8, 23, 43, 44, 70]. It has been suggested by Nebert [57] that an animal has the inherent or inducibly genetic ability to produce numerous different cytochrome P-450 proteins. The presence of different P-450 isozymes has been demonstrated in rat liver with the use of P-450 inducers, such as phenobarbital, 3-methylcholanthrene, B-naphthoflavone and 2-acetylaminofluorene [1, 2, 44]. Studies in human liver have demonstrated the existence of multiple P-450 isozymes for the metabolism of individual xenobiotics [39, 46]. To date at least six P-450 isozymes from human liver have been identified by their electrophoretic, spectral, catalytic and immunochemical properties [8, 70]. For the metabolism of 2-AAF, which we have studied extensively, experiments indicated that at least two [47, 48] and possibly three [1] forms of human liver P-450 are involved. However, these P-450 enzymes are most likely not the only systems which can oxidatively metabolize 2-AAF and it can be speculated that more P-450 isozymes and other enzyme systems will be identified.

1.3 GENETIC FACTORS AFFECTING CYTOCHROME P-450 XENOBIOTIC METABOLISM

Although factors such as nutrition, age, sex, geographical location and occupational exposure may contribute to the level and/or multiplicity of P-450 isozymes, genetic factors appear to be the most important [57]. This genetic variation is in part regulated by the Ah locus, which is multi-allelic and can mediate metabolic responses by altering P-450 isozyme levels causing wide individual variations [56, 58]. In addition to the genetics involved in P-450 isozyme induction, the constitutive levels of the isozymes must also be genetically determined.

It is important to identify genetic variability in the cytochrome P-450's, and determine which specific human P-450 phenotypes may be factors in the etiology of cancer on exposure to specific classes of chemical carcinogens, in order to predict individual sensitivity [35]. Slow and fast metabolizers of the model carcinogenic aromatic amine, 2-AAF, have been identified using human liver microsomes [53] and the data suggested that fast activators of 2-AAF were in most cases also fast detoxifiers. The hereditary differences in acetylator status between fast and slow acetylators has been shown [40] to be another metabolic event under genetic control. Individual susceptibility as well as target organ susceptibility to aromatic amine induced cancer and other toxic events may be affected by acetylator status [71]. The extension and clinical application of such findings may lend credence to the theory of diagnosing certain individuals as more or less susceptible to some chemically induced cancers.

It is also known that genetic polymorphisms exist in the oxidative metabolism of medicinal drugs. Some widely studied examples are sparteine, mephenytoin, debrisoquine, phenacetin, phenformin and bufuralol (Kahn *et al.*, [37] and references therein). Two phenotypes, the extensive metabolizer and poor metabolizer have been observed in human populations [17]. This has led to the discovery and understanding of therapeutic failures and increased risk of drug toxicity in the poor metabolizer group. As an example, a deficiency of a P-450 isozyme that causes the 4-hydroxylation of debrisoquine was found to be an inherited homozygous recessive trait associated with decreasing metabolism of sparteine and phenformin, and a decreased incidence of bronchial and G.I. tract carcinomas [38].

Genetic factors that cause variations in metabolism are apparent when looking at different ethnic groups [38]. Some classes of biotransformation reactions that have demonstrated variation are hydrolysis (cholinesterase and paraoxonase activity), conjugation reactions (glucuronidation, sulfation and acetylation) and oxidative reactions (P-450 and dehydrogenation, [38]). Some examples of these interethnic differences are given here. Caucasians exhibit an increased frequency of a structural variant of plasma cholinesterase compared to negroes and orientals (30 to 100 times higher, respectively). In Canada, white Canadians exhibit a 1:1 ratio of rapid to slow acetylation status, while Canadian Inuits are 90% fast acetylators. Europeans have a greater ability to form glucuronide conjugates of paracetamol, thus increasing its clearance compared to people of Asian extraction, but factors in addition to genetic differences may also contribute to this difference [38]. The genetic polymorphisms discussed earlier with P-450 isozymes for various drugs (sparteine, debrisoquine, etc.) also show ethnic variation as well as variation within heterogeneous populations.

From these examples, it can be seen that differences in the metabolism of xenobiotics are caused to a great extent by variations in genetic makeup. However, while these differences have been observed for specific drugs, it is too early to know how generally they can be extrapolated to other drugs. While increased activation (Phase I) combined with decreased detoxification (Phase II) of a chemical(s) would appear to increase carcinogenic (or toxic)

susceptibility, the outcome may vary with the specific chemical and further studies on the relationship of metabolism to carcinogenesis are needed to fully elucidate the generality of such an approach in aiding the prediction of human risk.

2. Experimental Hepatocyte Metabolism

2.1. SPECIES DIFFERENCES IN XENOBIOTIC METABOLISM

It is well documented that Phase I and Phase II metabolizing enzymes exhibit organ, species and individual differences in regard to substrate specificities and overall enzymatic levels [9, 10, 21, 42, 68]. The existence of multiple P-450 isozymes with unique substrate specificities are also subject to organ and species differences [24] and the isozyme differences may affect the sensitivity of a species or change the target organ within the species to the effects of a xenobiotic.

To date, most xenobiotic metabolism studies, other than whole animal studies, have been done with subcellular liver fractions. These preparations have been used in determining species differences in metabolism as well as acting as a metabolic activation system in short-term assays [6, 16, 28, 59]. However, the usefulness of subcellular preparations, such as S-9 or microsomes are limited when it comes to the determination of the *in vivo* metabolic fate of a chemical. Subcellular fractions cannot produce the secondary metabolic products that frequently are formed in whole animals. These preparations also do not contain some of the functional enzymatic systems or their cofactors, which act as competing activation/detoxification pathways that are present *in vivo*. To overcome some of these limitations of subcellular preparations, many studies of xenobiotic metabolism and of comparative species metabolism have been done using intact hepatocytes or other cell types [42]. Intact hepatocytes presumably mimic the *in vivo* liver metabolic behaviour better than liver S-9 or microsomal preparations, thus allowing a more complete determination of a chemical's metabolic fate. This is because hepatocytes contain all the mixed function oxidase enzymes that are present in subcellular fractions, in addition to all other enzyme systems functional in the liver *in vivo* [5]. A study by Green and co-workers [22], using intact hepatocyte suspensions from different species demonstrated that *in vitro* hepatocyte metabolism of amphetamine correlated with each species' *in vivo* metabolism. This correlation was not evident with S-9 or microsomal preparations, further emphasizing the value of intact hepatocytes for such studies.

2.2 SPECIES DIFFERENCES IN THE METABOLISM OF 2-ACETYLAMINOFLUORENE BY INTACT HEPATOCYTES

In hepatocytes, 2-acetylaminofluorene is metabolized by the P-450 enzyme system to ring hydroxylated products at the 1, 3, 5, 7, 8 and 9 positions, which can then be conjugated to water soluble glucuronide, sulphate or amino-acid (including glutathione) products [41, 61, 68, and references therein]. The N-hydroxylated intermediate of 2-AAF metabolism is believed to be the initial step in the conversion of 2-AAF to a mutagenic/carcinogenic form. This compound can be deacetylated to N-hydroxy-2-aminofluorene, a more

mutagenically active product than N-hydroxy-2-AAF [68, references therein]. A deacetylated product, 2-aminofluorene (2-AF) is also formed from 2-AAF and can then be N-hydroxylated to N-hydroxy-AF [3, 18]. Further evidence of the mutagenic activity of 2-AF and N-hydroxy-2-AAF is seen in mutagenesis studies where they are found to be more active than 2-AAF [50, 52, 69].

In the study by Langenbach and co-workers [41], rat, dog, and cow hepatocyte metabolism of 2-AAF was compared. The extent of 2-AAF metabolism to organic-soluble and water-soluble metabolites by hepatocytes from the three species is shown in Table 1. Cow hepatocytes showed the highest total metabolism followed by rat and dog hepatocytes, respectively. The difference in average total metabolism between cow and dog hepatocytes was almost two-fold. Within each species, the range in total metabolism was less than two-fold (data not shown).

The quantitative analysis of individual organic soluble metabolites produced by hepatocytes from the three species is shown in Table 2. 2-AF was the major metabolite formed by hepatocytes from all species. For cow hepatocytes, 2-AF accounted for over 80% of total organic-soluble metabolites, while

Table 1. Metabolism of 2-AAF to organic and water-soluble metabolites by hepatocytes from cow, dog and rat[a, b]

	Cow	Dog	Rat
Organic-soluble	33.7 ± 6.3	17.8 ± 4.8	20.2 ± 9.25
Water-soluble	21.3 ± 8.2	10.5 ± 4.2	14.4 ± 2.3
Total	51.0 ± 9.2	28.3 ± 6.3	34.7 ± 10.6

[a]The incubation time was 4 hour with 3×10^6 hepatocytes in 3 ml medium.
[b]The values represent the average of per cent 2-AAF metabolized for three bovine liver, three canine livers and four rat livers. The per cent water-soluble and organic-soluble radioactivity was based on the initial amount of [^{14}C]2-AAF added. The concentration of 2-AAF was 13.7 μM. Organic-soluble metabolism was determined after h.p.l.c. analysis and subtraction of the unmetabolized 2-AAF. Water-soluble metabolism was the radioactivity remaining in the aqueous phase after organic extraction.

Table 2. Hepatocyte metabolism of 2-AAF to individual organic-soluble metabolites[a]

Metabolite	Cow	Dog	Rat
	pmoles/hour/10^6 cells		
2-AAF			
7-OH	18–78	30–90	142–315
8-OH[b]	16–55	21–48	48–181
5/9-OH	40–59	150–449	12–50
3-OH	150–220	50–175	8–40
1-OH	52–214	127–194	49–212
N-OH	69–208	52–280	59–102
2-AF	2190–3710	505–1240	584–2400
Total	2530–4550	935–2480	902–3300

[a]Numbers are expressed as the ranges for the numbers of animals given in Table 1
[b]Radioactivity tentatively identified as the 8-hydroxy-AAF metabolite by h.p.l.c. cochromatography

Table 3. Metabolism of 2-AAF to water soluble metabolites by hepatocytes[a]

Metabolite	Cow	Dog	Rat
	pmoles/hour/10^6 cells		
Ring Hydroxylated			
Sulphate	46–166	15–107	364–496
Glucuronide	101–322	58–192	342–538
N-hydroxylated			
Sulphate	3.0–15	1.5–4.8	2.8–4.1
Glucuronide	14–77	3.8–1.3	4.2–18
2AF			
Sulphate	63–313	28–124	23–62
Glucuronide	119–243	33–134	31–57
Total	347–1136	139–575	767–1176

[a]See legends to Tables 1 and 2 for details

in rat and dog hepatocytes, it represented 64% and 50% of total metabolism, respectively. N-hydroxy-2-AAF was formed by all three species. Each species also formed a different predominant ring hydroxylated metabolite, thus illustrating species dependent P-450 isozyme variability in 2-AAF metabolism. Another point illustrated in Table 2 is the variation in ranges of individual metabolites. For example, with dog hepatocytes the range of N-hyroxy-2-AAF varied 5.4 fold, whereas the level of 1-hydroxy-2-AAF from the same animals varied less than two-fold. Hepatocytes from all three species were capable of conjugating 2-AAF metabolites to glucuronic acid and sulphate. Conjugation levels in the cow and dog varied about three- and four-fold, respectively (Table 3). For these species, the animals were of different age, sex and breed which may account for the variation. However, for the outbred Sprague-Dawley rats, which were all males of the same age and housed similarly, variations in total conjugation was only about 50%. Data from Table 3 shows that rat hepatocytes formed the highest levels of ring-hydroxylated conjugates, cow hepatocytes formed the highest levels of 2-AF, with dog hepatocytes being two to three times lower. Levels of glucuronide conjugation of ring hydroxylated products are higher than sulphate conjugation levels in all three species.

In a comparative study by Holme *et al.* [30], also using 2-AAF and cultured hepatocytes from hamsters, guinea pigs, mice and rats, 2-AAF metabolism was measured at various times and concentrations. Hamster hepatocytes produced the highest level of organic-soluble metabolites, almost two-fold greater than the other species. A wider species variation occurred in water-soluble metabolite formation with guinea pigs exhibiting greater than four times the level observed in rat hepatocytes. Detectable levels of N-hydroxy-2-AAF were observed only in hamster hepatocytes. Notwithstanding certain differences between our studies and Holme *et al.* [30], the combined results illustrate the species differences which can be studied using intact hepatocytes.

2.3 COMPARISON OF HUMAN AND RAT HEPATOCYTE METABOLISM OF 2-ACETYLAMINOFLUORENE AND BENZO(A)PYRENE

Since the data shown above illustrate metabolic differences between each

species and that *in vitro* metabolism can be related to *in vivo* metabolism [22], it can be hypothesized that hepatocytes from humans would be useful in predicting human liver metabolism. Furthermore, because the rat is a common test animal for potential human carcinogens, a comparison between rat liver and human liver metabolism of a known rodent liver carcinogen may be useful for understanding what the human response would be.

2.3.1. 2-Acetylaminofluorene

Although data exist on 2-AAF metabolism by rat hepatocytes, the only study to compare rat and human hepatocyte metabolism and mutagenic activation of 2-AAF in Rudo *et al.* [61]. Surgical and organ donor human tissues were utilized instead of autopsy tissue, as viable cells could not be obtained from autopsy samples and surgical specimens could be processed rapidly. Many previous human cell metabolism studies utilized autopsy tissue for explant culture of various tissues [4, 25, 27], but this source is not suitable for hepatocyte isolation. The slicing technique was used for hepatocyte isolation [61] and allowed for the preparation of hepatocytes from human surgical samples that were not perfusable. Thus, more individual human liver samples could be used with the viability and metabolic activation capability of hepatocytes prepared by this method, comparable to hepatocytes prepared via the more routinely used perfusion technique from the same individuals (Table 4).

Table 4. Comparison of *Salmonella* mutagenesis and viability of hepatocytes prepared by the slicing and perfusion technique

	TA98 Revertants			
	Individual I		*Individual III*	
AF Concentration (μg/plate)	Sliced	Perfused	Sliced	Perfused
Control[a]	18	21	34	24
1	63	97	77	58
10	203	182	258	156
25	298	250	279	240
Viability[b] (%)	92	90	82	73

[a]TA 98 revertant counts based on two plates/point at a cell concentration of 0.5×10^6 hepatocytes plate.

[b]Viability determined by trypan blue exclusion.

In Table 5, total metabolism by hepatocytes from eight individual human cases are shown. For comparison, data are also shown for male Fisher rat hepatocytes. Hepatocytes from Individual I had the highest overall metabolism (49.4%) of 2AAF and highest metabolism to water-soluble products (45.4%) with Individual IV exhibiting the lowest metabolism (19.8 and 7.3%, respectively). The range of conjugative metabolism by human hepatocytes was greater (7.3–45.4%) than the range of overall metabolism (19.8 to 49.4%). Except for Individual IV, human hepatocyte metabolism of 2-AAF to organic- and water-soluble products was greater than rat hepatocyte metabolism.

The ranges of specific organic-soluble metabolite levels formed by the hepatocytes are shown in Table 6. The major organic-soluble metabolites

Table 5. Metabolism of 2-Acetylaminofluorene by human and rat hepatocytes[a]

	Human								Rat[b]
	I	II	III	IV	V	VI	VII	VIII	
% Total AAF metabolized[c]	49.4	36.4	45.9	19.8	42.5	38.4	40.1	31.8	19.6 ± 3.3
% Watersoluble metabolism	45.4	23.2	28.0	7.3	16.5	19.6	8.2	19.8	8.1 ± 0.77

[a]All experiments were done of 4 μM [^{14}C] 2-AAF for 4h in flasks containing 5 × 10^6 cells
[b]Each human hepatocyte value represents one experiment in triplicate and rat hepatocyte values represent the average of three experiments
[c]The per cent 2-AAF metabolized and per cent water-soluble metabolism was based on the initial amount of [^{14}C] 2-AAF added. The total 2-AAF metabolized represents the per cent organic-soluble and water-soluble metabolism

Table 6. Metabolism of 2-AAF to organic-soluble products, and glucuronide and sulphate conjugated metabolites by human and rat hepatocytes[a]

Metabolite	Human[b] (pmoles/hour/10^6cells)	Rat[c] (pmoles/hour/10^6 cells)
Unknown		
Organic-soluble	2.2–45	28–35
Glucuronide	1.4–21	4.1–5.4
Sulphate	0.9–27	4.4–9.6
7-OH-2-AAF		
Organic-soluble	1.8–44	6.3–25
Glucuronide	1.4–49	7.4–16
Sulphate	0.9–29	9.8–16
8-OH-2-AAF		
Organic-soluble	3.9–18	2.0–7
Glucuronide	1.2–9	2.4–7
Sulphate	0.9–9	3.7–4
5/9-OH-2-AAF		
Organic-soluble	3.9–24	1.9–4
Glucuronide	0.5–3	0.7–3
Sulphate	0.4–4	0.7–2
1/β-OH-2-AAF		
Organic-soluble	4.3–25	7.2–19
Glucuronide	1.3–9	1.1–1
Sulphate	0.9–9	0.9–1
N-OH-2-AAF		
Organic-soluble	2.6–22	1.0–3
Glucuronide	1.1–14	1.0–1.5
Sulphate	1.0–16	0.9–4
2AF		
Organic-soluble	25–391	101–184
Glucuronide	13–128	20–39
Sulphate	10–111	250–43

[a]All experiments were done using 4 μM [^{14}C]-2-AAF for 4 hours. Hepatocytes were incubated with 5 × 10^6 cells in 5 ml medium.
[b]Values represent ranges of human hepatocyte metabolites from eight individuals.
[c]Values represent ranges of rat hepatocyte metabolites from three animals.

formed by both human and rat hepatocytes were 2-AF, 7-hydroxy-2-AAF and an unknown metabolite(s). The primary glucuronide and sulphate conjugated products formed by human and rat hepatocytes were the 7-hydroxy-2-AAF, N-hydroxy-2-AAF and 2-AF. It is interesting that while total metabolism and water-soluble metabolism with human hepatocytes varies two-and-a-half-fold and six-fold respectively, (Table 5), the range of individual metabolites such as 2-AF (organic-soluble) varied 35-fold (Table 6). This range for 2-AF formation may represent a genetic variation in the human deacetylase or be due to the acetylase variability. Likewise, the 7-hydroxy-2-AAF glucuronide and sulphate conjugates varied 30- and 36-fold (Table 6), respectively, which may indicate a genetic variability in these enzymes among the eight human cases. The range of inter-animal variation of specific metabolites by rat hepatocytes was significantly lower (less than four-fold) than human hepatocytes.

The data in Fig. 1 illustrate the metabolite levels from hepatocytes of each individual human and the average of three rat experiments and are shown for comparison. Carbon-hydroxylated metabolites are combined in Fig. 1 and data are presented as levels of organic-soluble and water-soluble (glucuronide and sulphates combined) metabolites. In humans, the major organic-soluble

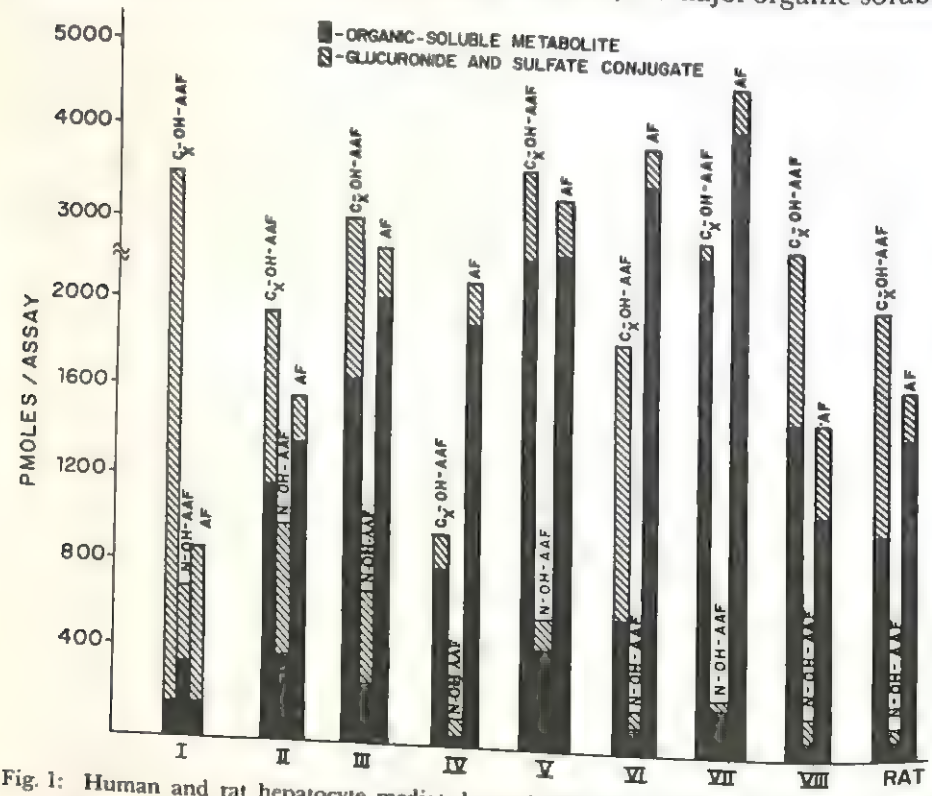

Fig. 1: Human and rat hepatocyte mediated metabolism of 2-AAF. Solid areas represent organic-soluble metabolites and cross-lined regions represent glucuronide and sulphate conjugate levels. CX-OH-2-AAF values represent the sum of all carbon-ring hydroxylated metabolites. Results are from individual human cases and the average of three rat experiments. All experiments were conducted for 4 hours, at a [^{14}C]-2-AAF concentration of 4 μM with 5 × 10^6 hepatocytes in 5 ml medium with three flasks/experimental point.

Table 7. Correlation between 2-AAF mutagenesis and 2-AAF metabolite formation by human hepatocytes

Individual	*2-AF Formation*[a]	*2-AAF Revertants/plate*[b]
II	80	187
IV	111	193
III	131	253
V	162	360
VI	195	455
VII	229	1225

[a]2-AF metabolized values are pmoles/hour/106 cells. 2-AAF initial concentration was 4 μM. Incunation time was 4 hours/using 5×10^6 hepatocytes/5 ml media.
[b]2-AAF revertants from *Salmonella typhimurium* assay. Concentration of 2-AAF/plate was 25 μg at a cell concentration of 1×10^6 hepatocytes/plate.

product formed was 2-AF (except Individual I) and the major conjugated metabolites were the combined Cx-hydroxy-2-AAF metabolites. Levels of N-hydroxy-2-AAF formed by human hepatocytes were variable and low compared to other metabolites. 2-AF formation was highest in Individuals V, VI, and VII and lowest in Individuals I and VIII. Levels of Cx-hydroxy-2-AAF formation were highest in Individuals I and V with Individual IV showing the lowest formation. By contrast to human hepatocytes, rat hepatocyte metabolism to Cx-hydroxy-2-AAF products were higher than levels of 2-AF formation. Additionally, the results with human hepatocytes suggested a role for deacetylation in the genotoxic effects of 2-AAF, as increased metabolism to 2-AF was associated with the increased mutagenesis of 2-AAF (Table 7). This relationship was not observed with rat hepatocytes and may suggest that for human hepatocytes, the further activation of 2-AF may be an important metabolic event in the genetic toxicity of 2-AAF.

Comparison of rat hepatocyte 2-AAF metabolism data in Tables 1 and 4 indicates that a higher level of metabolism was observed in Table 1. The rats used for the study in Table 1 were six to ten-week-old male Spraque Dawley used for the study in Table 1 were six-to-ten-week-old male rats. The rats used to obtain the data in Table 4 were six-to-ten-week-old male Fischer rats. Thus, the data may suggest a rat strain difference in the metabolism of 2-AAF, with Spraque Dawley rats being more active. Rodent strain differences in response to chemical carcinogens are well documented; however, in the present case the animals were housed in different facilities and with different diets. Thus, the exact cause(s) of the observed metabolic differences are unknown, but strain differences are a possibility currently under investigation.

2.3.2. Benzo(a)pyrene

Benzo(a)pyrene (BP) is a widely studied polycyclic aromatic hydrocarbon with mutagenic, carcinogenic, and teratogenic activity [19, 31]. BP metabolism is catalysed by P-450 oxidative enzymes and epoxide hydrolase [15, 19, 20, 63]. BP is hydroxylated at various carbon positions during metabolism and metabolites are frequently converted to glucuronic acid, sulphate or glutathione conjugates [62, 65]. BP metabolism was investigated in rat hepatocytes [64] and in human hepatocytes by Moore and Gould [55]. In both the studies,

the major metabolites were the 9, 10-diol, 7,8-diol, 9-phenol and 3-phenol with trace levels of pre-9, 10-diols and quinones. Rat hepatocytes metabolized BP to 30% water-soluble and 70% organic-soluble metabolities while human hepatocytes exhibited a range of water-soluble metabolism of 76 to 96% and organic-soluble metabolism of 4 to 24%. The inter-individual variation among six human cases in specific BP metabolite formation was much greater than the variation in total organic or water-soluble metabolism [55]. For example, while total BP metabolism ranged from 36 to 91%, the ranges of specific metabolites were 6 to 48% for pre-9, 10-diols, 8 to 45% for 9, 10-diol, 6 to 20% for the 7, 8-diol and 11 to 42% for the phenols. From these comparisons of human and rat hepatocyte metabolism of 2-AAF and BP, as well as from the species comparisons illustrated earlier, it can be seen that quantitative differences rather than qualitative differences between these species exist. It should also be noted that 2-AAF and BP can be metabolized via several pathways and lower activities in one pathway may be compensated by higher metabolism via another pathway. Thus quantitative differences in total metabolism can be much smaller than quantitative differences in individual metabolites. However, for chemicals that have only one pathway of activation, even quantitative differences in that pathway could have profound effects on the chemical's activity.

3. Utility of Human Hepatocyte Data in the Investigation of in vitro Metabolism of Xenobiotics

For many years, animal studies have been widely utilized to provide data on carcinogenic mechanisms and to assist in assessments of chemical risk to humans [31, 32]. However, species differences in carcinogenic responses are well-documented and can make direct rodent to human extrapolation difficult. Earlier on, such differences were not considered so significant, when evaluating human risks [13]. While many factors contribute to the differences in species response, a major factor is believed to be due to differences in the chemicals' metabolism. Furthermore, in the human population large individual differences are present, which further complicate extrapolation or rodent test data to the human population. As discussed above, while many species, strain or inter-individual metabolic differences are quantitative, some can be also qualitative. Thus, it is extremely important to know of such differences when extrapolating from the test species to the human population.

In the present work, we have discussed how human hepatocyte metabolism of 2-AAF and BP compares with their metabolism by hepatocytes of other species. It is hoped that by identifying these metabolic differences and understanding the genetic basis for them, that extrapolation of carcinogenesis data from these species to the human will be facilitated. However, a caveat to this process is that, at present the relationship between metabolism (i.e. identified metabolites) and carcinogenesis is not entirely clear. This is especially true for chemicals with multiple pathways of metabolism. While the metabolism of carcinogens and the genetic basis controlling it are not the only factors involved in the carcinogenesis process, it is believed that

increased understanding of species differences in a chemical(s) metabolism combined with a knowledge of human cell/tissue metabolism will aid in predicting chemical effects in humans.

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4

Recent Developments in the Cellular Genetics of the Skin

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1. Introduction

As in most other areas of biology, cellular genetics is now almost synonymous with DNA technology. Our skin is the most accessible organ which we have, and we are the most numerous large animal species. Many genetic disorders of the skin have been described, the so-called genodermatoses. The protein biochemsitry of these has only been studied with moderate success so far. However DNA probes for collagens and keratins and many other skin components are now available and since these can be used with the DNA extracted from relatively minute biopsies, the prospect is that dermatology, until recently an almost totally descriptive discipline, is likely soon to become one of the most scientific.

2. Genetics of the Major Structural Components of the Skin

2.1 THE EPIDERMIS AND KERATINS

The basal layer of the epidermis is made up of rapidly dividing cells which receive their nutrition from the underlying dermis. Above this layer is the stratum spinosum of the rete Malpighii, then the stratum granulosum. These names indicate a gradual change of character of the cells as they mature to produce the stratum corneum, the outermost, partially watertight, dead layer which confronts the chemical and physical world and is mainly composed of keratin.

Keratin [106] is a fibrous protein found in all layers of the epidermis, in the form of tonofilaments about 8 nm in diameter. These filaments are intracellular and are continuous from cell to cell via attachment plaques in the cell membranes. The cells are linked by desmosomes [13] and the attachment plaques are part of the desmosome complex. Other bodies in the epidermis are the keratohyalin granules in the granular layer [12] and the membrane coating granules in the upper spinous layer [65]. These latter granules, which have a significant lipid content [35], disintegrate at the base of the stratum corneum to give rise to lamellar sheets.

Fuchs *et al.* [41] have reviewed the basis of the keratin gene expression. The 8 nm filament has been found to be composed of a number of smaller closely packed filaments of about 2 nm diameter, the protofilaments. Each protofilament is composed of several helical polypeptide strands intertwined in a coiled-coil configuration. Cloning and equencing of cDNAs complementary to mRNAs, and protein chemical studies, have enabled the establishment of 50 kd type I and 56 kd type II keratin structures. There is less than 30% amino-acid homology between types I and II. Despite such low homology, the predicted secondary structures are almost identical. Thus the two types may interact to produce the coiled-coil structures. Basically each molecule is a helical rod with four components of fixed size and end domains which are variable according to the differentiations found in different anatomical sites including the skin. Type I keratins have end zones with two components, while end zones in type II have three. More specialized but related proteins, e.g. vimentin, desmin, glial fibrillary acid protein (GFAP) and neurofilaments, each have their specialized end zones. The four rod

components are named 1A, 1B, 2A and 2B, each 35, 101, 19 and 121 amino acids in length, respectively. To separate these four segments there are three non-coiled-coil linkers each of less than 20 amino-acids. Each α-helical rod is composed basically of seven amino acid quasi-repeats (heptads). In the middle of component 2B there is a reversal of polarity of the heptads producing an irregularity in the coiled-coil at that point.

At least 20 different keratins have now been identified by size and charge differences on two dimensional gel electrophoresis [69]. This suggests at least 20 keratin genes [54]. So far eight DNA clones have been obtained containing part, or all of more than one keratin gene, suggesting extremely close linkage between different genes [9]. This is analogous to what is found with the histocompatibility genes and is quite different from current observations on the collagen genes. Three clones contain both type I and type II genes. Some clones contain genes for keratins with other distinctions, e.g. the glycine-rich keratins and those which are not glycine-rich. Most of the genes may be separated by no more than 8 to 10 kbp of DNA.

Tyner *et al.* [131] have reported that there are eight introns in the gene for the 56 kd type II keratin. This is closely similar to the case of vimentin, which contains a type III protein analogous to the types I and II (type III intermediate filament, IF). Thus types I, II and III IFs must all have a close evolutionary origin. All the highly conserved intron positions are within, but do not demarcate, the four α-helical domains which are characteristic of all IFs.

So far the keratins, being the predominant proteins in the skin, have been the most studied. Clinically, the keratohyalin granules of the stratum corneum have importance. A major protein component of these granules is filaggrin (filament aggregating protein). Prelimanary work [66] in studying the protein by gel electrophoresis, and in isolating a mRNA (in rodents), which is large enough to code for a 600 kd polypeptide, suggests that filaggrin is synthesized as a large MW precursor, which is substantially processed to produce the final protein product.

Fleckman *et al.* [39] have reported on the post-translational modification of pro-filaggrin to filaggrin. In cultured keratinocytes a high M_r precursor is produced, but the processing to a lower M_r product does not occur. Kubilus *et al.* [59] studied the filaggrins using human and rat specific antibodies. In both species profilaggrin was produced, but in culture only the rat protein was further processed.

2.1.1 Genetic disorders of the epidermis

2.1.1.1 Ichthyosis vulgaris: Sybert *et al.* [127] studied this autosomal dominant disease. In two families filaggrin was absent from the skin of the most severely affected individuals. Keratohyalin granules were absent also in the three worst cases and reduced in a number of others. The amounts of keratohyalin and of filaggrin corresponded.

2.1.1.2 X-linked ichthyosis: Crawfurd [26] has reviewed the genetics and biochemistry of this disease, which affects about one person in 6,000. The mothers of affected males were noted to have low levels of oestrogen with

failure of onset of labour and inadequate dilatation of the cervix. The affected sons were deficient in steroid sulphatase. Hoyer *et al.* [49] have reported a detailed clinical study of 76 males with the enzyme deficiency. Regular ichyhyotic scaling was usually heralded by a general peeling of the skin. Those infant boys idenfitied by a steroid sulphatase deficiency in the placenta showed signs between one and three weeks of age. Polygonal scales were noted later, on distal parts of the body and extensor aspects of the extremities. Sever *et al.* [111] have reported on corneal opacities, which also develop in time.

Okano *et al.* [87] have studied heterozygote detection, using steroid sulphatase levels in lymphocytes. All affected males had absent enzyme, and many females related to them had significantly lower levels than normal controls. This might be expected on the basis of random inactivation of the X-chromosome. Lykkesfeldt and Lykkesfeldt [64] found that steroid sulphatase assays carried out on leukocytes and skin fibroblasts enabled the identification of 30 of 31 heterozygous females. They discuss the need for identification of the heterozygotes, the prenatal diagnosis of affected boys, and the harmful effects of the gene on organs or tissues other than the skin.

Teipolo *et al.* [129] located the gene for the disease at Xp 223 by deletion mapping. It has been suggested that this area of the X-chromosome is not subject to X-inactivation [114]. The locus is 10 cM from that of Xg, which has also been suspected of not being inactivated [68]. Muller *et al.* [70] reported that the fibroblasts of normal females had 1.7 times the level of steroid sulphatase as did those of normal males. Some carrier females are mildly ichthyotic, which supports inactivation of the locus, but no clear decision about inactivation is yet forthcoming.

2.1.1.3 Lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE): The broad details of the classification used here suggest that the principal autosomal recessive type of ichthyosis is LI. Genetic disease has a habit of gaining in complexity, the more it is studied, and Bernhardt and Baden [8] have reviewed 42 cases which do not all fit neatly into the traditional categories of LI and CIE. They feel that the autosomal recessive diseases may be quite heterogeneous. Williams and Elias [137] acknowledged the heterogeneity but claimed that LI and CIE are still clearly definable and distinctive categories.

Rossman-Ringdahl *et al.* [105] have described a mother and two children with CIE. They refer to the pattern as pseudo-dominance, and suggest that LI and CIE are heterogeneous. There is often consanguinity in families with these rare diseases, but there was none in the family described.

Li is a rare (1 : 300,000) condition, which is observed at birth, the child being encased in layers of stratum corneum giving rise to the name, collodion baby. The material is shed freely and is thinned out within a few weeks of birth. Microscopically, there are increased keratinosomes, with mitochondria and nucleated cells in the upper granular layer [132]. This could result from increased mitotic activity and reduced cell transit time from basal layer to stratum corneum. The defect may be in the cellular dynamics of the epidermis.

2.1.1.4 Bullous ichthyosiform erythroderma (BIE): BIE also occurs in 1 : 300,000 live births, but is autosomal dominant. The bullae are common in childhood, but become less so in adulthood. Large clumped keratohyalin granules are present in a thickened granular layer. There are large areas of perinuclear endoplasmic reticulum, abundant canaliculi, ribosomal particles and mitochondria in the cells of the granular and upper Malpighian layers [136]. The relationship between tonofilaments and desmosomes is abnormal. This may enable the accumulation of fluid beneath the stratum corneum leading to vesiculation.

2.1.1.5 Harlequin foetus: The most abnormal ichthyosiform condition is harlequin foetus. The frequently premature baby is enclosed in hard plates of thickened cornified epidermis. The condition is rapidly fatal. Baden and Goldsmith [4] demonstrated the presence of an abnormal keratin in one case. The condition is autosomal recessive.

2.2 THE DERMIS AND COLLAGENS

The genetics of the dermis relates in great measure to the genetics of the collagens, which will be briefly reviewed. Additionally, there are diseases arising from defects at the dermo-epidermal junction and it appears that these often arise as a result of defects in collagen rather than keratin.

Adjacent to the epidermal basement membrane there is a loose connective tissue network of thin collagen fibres, the papillary layer. Deep to this layer is a dense connective tissue layer, the reticular layer, with bundles of thick regularly oriented fibres. Deeper still is a fatty layer traversed by less organized collagenous fibres. The collagens in the dermis are types I, III and V, while types IV and V are found in the basement membrane.

Type I collagen has two pro—α_1 (I) chains and one pro—α_2(I) chain, each of which comprises terminal propeptides and a central domain of 338 Gly-X-Y repeats. In comparing the human pro—α_1 (I) and chicken pro—α_2 (I) Chu *et al.* [20] have made certain observations. The exon arrangement of the two genes is remarkably similar, although the human gene is shorter, having shorter introns. The structure of the chicken pro—α_2 (I) has a relationship between exon arrangements and Gly-X-Y repeats, suggestive of the possibility that the collagens evolved from a common ancestral unit of 54 base pairs. The complete sequence of human pro—α_1 (I) suggests evolutionary pressure to maintain a basic multi-exon pattern interspersed with 50 introns. These 50 introns add up to 12 kbp, which is two-thirds of the total gene size of 18 kbp. The triple helical domain of the pro—α_1 (I) gene codes for 996 amino acids, the codons being distributed in 41 exons each of 54 bases or multiples of 54. In addition to the above, there may be pro—α_1 (I) trimers present in the skin.

Weber *et al.* [133] studied the several specified layers of the dermis by biochemical analysis and immunofluorescent labelling with specific antibodies. The type III/type I ratio was lowest in the reticular layer. The amounts of type V collagen and pro—α_1 (I) trimers were constant in all layers.

There are at least three enzymes which convert pro-collagen to collagen before the latter can be properly aligned to produce functional fibres. There are two aminoterminal proteinases, one of which cleaves procollagens I and

II while the other cleaves procollagen III. A less specific carboxyterminal proteinase has also been isolated [94]. Lysine modifies the effect of the carboxyterminal proteinases in propeptides I and II, hence the possibility of more than one enzyme. Weber *et al.* [133] report that the degree of hydroxylation of lysine in types I and III collagens is the same in all layers of the skin.

The basement membrane below the epidermis contains types IV and V collagens, the glycoprotein laminin and heparan sulphate proteoglycan (HSP) [110]. Further investigations of protein relationships at this level are reported by Woodley *et al.* [139]. After separation of dermis and epidermis, a protein, bullous pemphigoid antigen (BPA), remains associated with the basal layer of the epidermis, while laminin, HSP and collagens IV and V separate with the basement membrane zone (BMZ)—basal cell plasma membrane, lamina lucida, lamina densa and sublamina densa. Laminin is found in the lamina lucida along with BPA. The sublamina densa contains anchoring fibrils.

The human collagen loci which have been identified so far are widely scattered in the genome, unlike what appears to be the case for the keratin genes. α_1 (I) is on chromosome 17, α_2 (I) on 7, α_1 (II) on 12, α_1 (III) on 2 and α_1 (IV) on 13. Type I and type II collagen genes are present in single copies. Fragment length variations have been observed with several restriction enzymes.

2.2.1 The collagenoses

Cheah [18] has provided a detailed review of this interesting and complex area. One condition, with minimal relevance to the skin, is osteogenesis imperfecta (OI). The most serious clinical feature is multiple bone fractures; poor development of the fibrous tissue of the sclera may lead to the physical sign of blue sclerae, which may be a useful sign in type I OI. In this type, there is a non-functional α_1 (I) chain and a shortened α_2 (I) chain. The condition is autosomal dominant. In Ehlers-Danlos syndrome (EDS) type IV there is thin, translucent and fragile skin, visible veins and easy bruising. There is absent or diminished type III collagen, possibly due to an amino-acid insertion in the α_1 (III) chain. In EDS VII there is extreme joint laxity, soft but non-fragile skin. There is apparent persistence of N-propeptide in 50% of pro—α_2 (I) chains. This could be due to a mutation affecting an N-proteinase recognition site. In Marfan's syndrome, there is basically no skin disorder. The subjects have very long thin legs, arms and digits with increased flexibility of many joints. Dislocation of the lens of the eye is common.

It seems likely that in the long run the molecular variations amongst collagens will be as great as those amongst haemoglobins. Fibroblasts can be grown from the amniotic fluid and it is possible to detect type I and type IV collagen synthesis in these cells. Unfortunately, type II is not expressed in fibroblasts.

There is an additional complicating factor in relation to one of the accepted collagenoses—Yamakage *et al.* [141] have described three cases of classical EDS, in which they could find no abnormality of the collagen, but in which they found a hyaluronic acid-dominant glycosamino-glycan structure

in the intercellular matrix. There was significant increase in hyaluronic acid, and skin fibroblasts synthesized more than usual amounts of hyaluronic acid in culture. Such cases may make the ultimate understanding of these diseases more complicated, but also more meaningful.

2.2.2 Epidermolysis bullosa (EB)

There are several diseases which have similar but not identical clinical manifestations. Some are autosomal recessive, some autosomal dominant. For a classification, one must go to a major text, like Fitzpatrick *et al.* [37]. Understanding at the cellular and molecular levels is coming slowly. Tidman and Eady [128] have reported on the heterogeneity of the hemi-desmosomes which are found in the basal plasma membranes of the basal cells and appear to have an important role in binding the basal cells to the underlying basal lamina of the dermis. Woodley *et al.* [138] and Paller *et al.* [89] have reported on the character and distribution of the epidermolysis bullosa acquisita (EBA) antigen. EBA is a serious bullous disease. The antigen is detectable in the basement membrane zone of the skin using labelled antibody. Western blot analysis by monoclonal and polyclonal EBA antibodies has revealed a protein of 800,000 MW, which has at least two chains, a major one of 290,000 MW and a minor one of 145,000 MW. These antigens are recognized by serum auto-antibodies in the developed disease. Both EBA antigen chains contain carbohydrate and the 290 kd chain is sensitive to collagenase. Gammon *et al.* [43] have described antibodies found in three patients with systemic lupus erythematosus (SLE), which were similar to EBA anti-basement membrane zone antibodies. They suggest that such antibodies represent part of an autoantibody spectrum for SLE.

Oakley and Priestley [85] have reported on an increased collagen synthesis in six patients with EB simplex and six patients with EB dystrophica. Cultured fibroblasts had increased collagenase activity, which may relate to the increased collagen synthesis and an involvement of the dermis in some types of EB.

2.3 THE MELANOCYTES AND MELANIN

Skin colour variation between different races has a deep emotional overlay, despite the obvious survival value of darkly pigmented skin to that large proportion of mankind who live in tropical and subtropical climates. Teleologically, the placement of the melanocytes at the dermo-epidermal junction and their ability to inject melanin granules into the basal cells of the epidermis is an elegant adaptation. The melanin can absorb ultraviolet light which has the potential to kill dividing cells rapidly or make them cancerous more slowly. The melanocyte has much in common with nerve cells structurally, and it is the dendrites which extend into the epidermis which have the cytocrine function of direct transmission of melanin granules into the cytoplasm of other cells. It is also relevant that dopa is a component of melanin and of the neuro-transmitter dopamine, so that the relationship to nerve cells exists at the biochemical level also.

The cellular genetics of melanogenesis resolves into biochemical aspects and general functional aspects of the melanocytes including the structure and

function of the dendrites and the structural variations among the melanosomes, which are the subcellular bodies in which the melanin is synthesized.

Biochemically, we have some generalizations about tyrosine to dopa to melanin pathways, but few definitive ideas as to the controlling mechanisms for the production of phaeomelanins (red and yellow pigment) and eumelanins (brown and black pigments). The latter have a lower level of cysteine incorporation [93]. Nicholls [77] did a simple experiment using equimolar dopa and cysteine reacted with ferric chloride. At pH5, the pigment produced had several apparently low molecular weight components, identical with pigments extracted from the hair of the Australian grey possum, which has a red-brown chest patch, and from feathers of red domestic fowl. At pH6 the pigment produced was brown and at pH7 it was black and precipitated rapidly from the reaction mixture. The conclusion, supported by work of Prota and Nicolaus [96], was that at slightly acid pH(5), dopa and cysteine were reacting to produce cysteinyl-dopas which were bright-red in colour, at pH7 cysteine was more likely to dimerize to cystine and dopa to polymerize to black melanin, while at pH6 intermediate results giving brown pigment were likely. The ultrastructural counterpart to these results is, perhaps, seen in the laminated character of the melanosomes.

Regarding the activity of the melanocytes and dendrites, there is some evidence from pigmentation studies in mice and other species. At the development stage, the melanocyte is a migratory cell which has to travel some distance from the neural crest in the developing embryo, in order to populate the whole skin surface. Furthermore, there is a need for melanocytes to enter and remain in the hair follicles, which are constantly being renewed as the hairs are shed. Patchy depigmentations and the occasional premature whitening of the hair in a disease like Waardenburg's syndrome, may be due to an abnormality in the behaviour of the melanocytes. In mice, there are genetic variations which may relate to structural and functional problems in the dendrites or elsewhere in the melanocytes. The *dilute* and *leaden* phenotypes may involve such mechanisms [37].

Finally there are well documented aspects of melanosome structure and function relating to normal and abnormal variation. Eumelanin producing cells have elongated oval shaped melanosomes with a clearly visible internal laminated structure, when viewed at an early stage of melanization. It is interesting that the melanosomes of oculocutaneous albinism have this structure, but without any sign of melanin being produced. Meanwhile, phaeomelanin producing melanosomes are usually rounder and less orderly in internal structure [93].

Many variations in the melanosomes have been described in disease states, e.g. tuberous sclerosis, neurofibromatosis, Chediak-Higashi syndrome, X-linked ocular albinism. Some of these will be considered later. Jimbow and Horikoshi [52] have discussed macromelanosomes which are found in the last three conditions named.

Looking at pigmentation genetics in a more general way, one may first consider the great array of species which have been domesticated and selectively bred for particular coat colours. Skin pigment variation in man does

not segregate in a Mendelian fashion. Hair pigmentation of many species does do so, but these are all highly inbred and selected animals. Workers have used reflectance spectrophotometry to get quantitative values of skin colour in man and from such data estimates of numbers of genes contributing to human variation have been made. The number of loci does not appear to be great, with a minimum estimate of three.

Nicholls [75, 78] developed an alternative approach to the study of the genetics of pigmentation from phenomena associated with freckles and pigmented birthmarks. The hypothesis was that these were due to mutation of a normal (inhibitory) pigmentation gene in a single cell, which once mutant at the specific pigmentary locus, would by conferring a proliferative advantage on the mutant cell, give rise to a clone of pigment cells, either a pigmented birthmark (effect before birth) or a freckle (effect after birth, most often but not always due to sunlight). On the basis of progressive pigmentary changes observable in many freckles, birthmarks and eyespots (iris nevi), he [81] was able to conclude that at least three gene loci were necessary and sufficient to explain the total normal pigment variation of the human species. The more or less infinite variations on the basic pattern can be explained on environmental factors and minor normal gene, or major pathologic gene, effects.

Polypeptide mediators of melanogenesis have been studied in detail in a number of animal models. Edelstein and Pawelek [34] have reported on studies of melanocyte stimulating hormone (MSH) receptors on the surface of melanoma cells. They claim that biotinylated MSH probes are 10,000–100,000 times more sensitive at detecting these receptors than corresponding radiolabelled probes and that these probes can detect two proteins of MW 68,000 and 120,000 in protein homogenates of melanoma subjected to gel electrophoresis. With this degree of senstivity of assay, genetic variations will ultimately be detectable at this level.

Already the origins of the two MSH hormones have been traced in some detail using DNA methods. Nakanishi *et al.* [72], using bovine pituitary glands, extracted the mRNA and produced double stranded cDNA, which was cloned in *E. coli*. The cloned cDNA inserts contained nucleotides which corresponded to the complete amino-acid sequence of corticotropin and the first part of β-lipotropin. These hormones which are polypeptides of 39 and 91 amino acids respectively, are parts of a much larger molecule. The two melanotropins, α-MSH and β-MSH, are found within the larger peptides of corticotropin and β-lipotropin respectively. Boileau *et al.* [10] prepared RNA from porcine pituitary intermediate lobes, and this was translated in a cell-free rabbit reticulocyte system. This produced two translation products which could be precipitated by a β-MSH antiserum.

2.4 THE IMMUNE CELLS OF THE SKIN

It is only recently that interest in the skin as an active part of the immune system has been possible. Some disorders with a suspected immunological involvement may also have a familial bias, but this is not usually strong enough to lead to a suggestion of Mendelian inheritance.

Recently the ability to unequivocally identify T-cells in the skin in associ-

ation with Langerhans cells [108] has been a significant step forward. The availability of cDNA codes for interleukins (ILs) and interferon (IFN) have provided a great impetus for new and critical studies.

Gubler *et al.* [45] have reported on the cloning of genes for IL-1. This polypeptide is produced by activated macrophages and is involved in many immunological responses. A murine IL-1 cDNA was cloned in *E. coli.* It encoded an IL-1 precursor of 270 amino acids, the carboxyterminal 156 amino acids of which was biologically active as IL-1. The murine cDNA was used as a probe to identify its human counterpart, nucleotide sequence analysis of which predicted a polypeptide of 271 amino-acids (IL-1α) with approximately 61% homology to the mouse protein, but with only 27% homology to a previously identified human IL-1 precursor (now called IL-1β). Purification of the mouse and the two human proteins has shown great similarity of activity despite the amino-acid differences.

Fujita [42] has reported studies on IL-2. Using a cDNA for human IL-2 which he and his colleagues had prepared, and cloned human and mouse IL-2 genes, it was found that each gene was contained within 7 kbp fragments, and each had three introns. The sizes of introns and exons in each species were comparable. There was notable homology upstream from the TATA box, indicating the likelihood of a regulatory sequence in that region. Yoshie *et al.* [142] reported on the use of IL-2 mass produced in *E. coli* following the recombining of cDNA from a human source. The mechanism of action of IL-2 is inadequately understood. They showed that the human IL-2 increased the level of production of 10 polypeptides in an IL-2 dependent murine cell line.

Danner *et al.* [28] report on an IL-3 like mediator produced by epithelial cells, which induces the proliferation of IL-3 dependent host cell lines. This mediator also increases the activity of natural killer (NK) cells, suggesting that a previously described epithelial cell derived natural killer cell augmentary factor (ENKAF) might be identical with IL-3.

2.4.1 Immune disorders affecting the skin

Disorders which often have a familial tendency include general and discoid lupus erythematosus, some cases of Raynaud's disease, systemic scleroderma divided into diffuse scleraderma (DS) and the CREST syndrome (calcinosis, Raynaud's disease, oesophageal dysphagia, sclerodactyly and telangiectasia). Chen *et al.* [19] have reported on parients with antinuclear antibodies in the above categories. Twenty-two patients had anticentromere antibodies. Patients with linear scleroderma [36] were found to have antibodies to double stranded DNA (ds DNA), while in seven patients there were antibodies to single stranded DNA (ss DNA). Antibodies are also often found directed at RNA, and in particular the uracil bases of nucleolar RNA.

Recently antibodies of another distinctive type directed at nucleic acids have been described. Two antibodies named anti-Ro and anti-La by one group, and anti-SS-A and anti-SS-B by another, have been defined. Ro and La are the first letters of patients' family names and SS stands for Sjogren's syndrome. These antibodies have been identified in a number of diseases, e.g.

neonatal lupus erythematosus, some rheumatic diseases, monoarthritis and fulminant lupus erythematosus. The antibodies are directed at a population of minute cytoplasmic particles composed of RNA and protein. Patients with anti-Ro/SS-A antibodies frequently had anti-La/SS-B as well, but were more often negative for anti-nuclear antibodies [62, 134, 135].

3. The Cellular Genetics of Neoplasia of the Skin

3.1 INTRODUCTION

In this section, the word neoplasia is used in its original derivation of new growth, so that a condition such as the common pigmented freckle in which cells appear in the skin in clonal fashion, where they were not previously present, qualifies as neoplasia. At the other end of this particular scale of course is the highly lethal malignant melanoma.

The effects of ultraviolet light (UVL) will be discussed in this introduction, because neoplasia of the skin cannot be validly considered without knowledge of this mutagen. Other mutagens of course affect the skin and one of the first links between an environmental agent and cancer ever to be observed was that between scrotal cancer and soot in chimney sweeps, in which some of the effect at least has subsequently been attributed to carcinogenic hydrocarbons in the soot.

Allied to the consideration of UVL is the extensive knowledge of DNA repair in human cells, which has accumulated since 1968. There is experimental evidence that vast numbers of mutations can occur in skin cells as a result of UVL exposure and these mutations can rapidly cause the development of lethal cancers, if there is deficiency of a specific repair process capable of eliminating UVL damage to DNA.

3.1.1. The effects of UVL on human skin

The most common effect of UVL on human DNA is to produce dimers between pyrimidine molecules which are adjacent to each other in the DNA chain. The most common dimer arises between pairs of thymine molecules to produce cyclobutane dimers. The formation of such a dimer necessitates the disruption of the pairing of the bases with the complementary DNA chain and, if left unrepaired, prevents the transcription of a mRNA capable of being translated to a functional protein, or the replication of the exact DNA sequence as it existed before the mutation.

The majority of people have a full complement of enzymes capable of repairing UVL damage to DNA. Those rare individuals who are homozygous for a recessive disorder, which leads to the absence of a repair enzyme usually have an extreme sensitivity to UVL, both in its sunburning and in its carcinogenic capacities.

Bartram [6] has reviewed knowledge of DNA repair pathways and defects. There are three major possibilities: photoreactivation, post-replication repair and excision repair.

In photoreactivation, which is not known to be important in man, a DNA photolyase binds to the cyclobutane dimers and produces a reversion to monomers in visible light without the need for an excision process. Suther-

land [125, 126] has isolated a photolyase from human cells and has found cases of xeroderma pigmentosum (XP) with a deficiency of this enzyme.

In post-replication repair, which becomes important if the DNA replicates before excision repair has occurred, gaps are left in the newly synthesized DNA molecule opposite where the dimer has been produced by UVL. Replication then resumes beyond the defect, and the gap is filled later by *de novo* synthesis. However, the dimer in the parent strand has not been excised, so that repair has not occurred. Caffeine and theophylline, purine analogues, are able to occupy the gaps left in the chain before *de novo* synthesis has occurred, which makes this repair system very sensitive to these agents. Some XP variants show a deficiency in this system [60].

The third mechanism is the most important, excision repair. Initial simplicity has given way to complexity. The molecular mechanism involves: (1) recognition of the error; (2) distortion of nucleoproteins; (3) incision of the damaged strand by endonuclease; (4) removal of the strand by exonuclease; (5) repair of the deficiency by polymerase, using the complementary strand as template; and (6) joining the cut ends of the repaired chain with a ligase.

There are other aspects of repair to add to the complexity: (1) nucleotide repair : recognition of dimers or carcinogen-DNA adducts, with excision; (2) base repair : removal of damaged bases by an N-glycolase and subsequent hydrolysis of the phospho-diester chain with apurinic endonucleases; DNA-DNA cross linking requiring base excision and nucleotide or base excision repair; and (4) single strand repair in which single strand breaks are prepared for subsequent repair and ligation using a special endonuclease.

Freeman *et al.* [40] have considered the minimum erythemal dose (MED) in relation to doses of UVB. A UVL endonuclease from *Micrococcus luteus* recognizes pyrimidine dimers in DNA, and the number of sites attacked by this enzyme can be estimated from alkaline agarose gel electrophoresis. Skin biopsies were taken from seven subjects after UVB irradiation in doses from 0 to 180 mJ/cm^2. The slopes of the dose response curves for the most UVL sensitive individual (MED = 24 mJ/cm^2) and the least sensitive (MED = 146 mJ/cm^2) were respectively 11.5×10^{-4} and 2.6×10^{-4} sites per 1,000 bases per mJ/cm^2.

Lin *et al.* [63] have studied an aspect of UVL repair in the skin—the role of solar conditioning in the repair response and the survival of keratinocytes following irradiation. The repair process appeared to be the same in sunlight exposed and protected areas. However, growth and survival of cells following dosages of 50 J/m^2 at 254 nm were greater in the protected keratinocytes than in the regularly sunlight exposed. Growth and survival were also greatly reduced in the keratinocytes of aged donors. These results can be understood if one assumes accumulation of sites of damage within the cellular genomes with sunlight exposure and with age which renders the cells more easily damaged by any increment of damage to the DNA.

UVL may play a major role in modifying the activity of the immune system in the maintenance of the integrity of the skin, particularly the epidermis. Nicholls [82] observed the regression of pigmented moles (melanocytic naevi) from sunlight-exposed areas of the skin before they regressed

from covered areas. The same applies to neurofibromata, which are derived from cells of the neural crest as are moles. There is circumstantial evidence that this regression is due to cell mediated immunity (CMI). UVL may be able to modify the production of immune and other mediators in the skin, e.g. IL-1, IL-2, IL-3 and interferon (IFN). It may also be involved in the cellular diversification of the lymphocytes of the skin—the rearrangement of the DNA which is known to lead to the diversification of immunoglobulins, and presumably also T-cell receptors. If UVL is changing the character of keratinocytes and melanocytes in the skin by a mutational process, there is no reason why it should not be changing the character of immune cells as well which are known to be present in the epidermis in great numbers [108].

Kripke and collaborators have reviewed progress up to 1981 on UVL carcinogenesis and reported on the distant effects of UVL in mice, in areas other than that directly irradiated. At the site of irradiation, there is an accumulation of leukocytes and a suppression of delayed type hypersensitivity and graft-versus-host reactions. Effects at a distance from the irradiation may be due to a soluble mediator and are seen as suppression of contact hypersensitivity and the accumulation of antigen specific suppressor T-cells [57, 58].

3.1.2. Xeroderma pigmentosum (XP)

This is a rare autosomal recessive disease (or group of diseases) in which there is an excessive sensitivity to UVL with the development of every type of malignancy in exposed cells. In the De Sanctis-Cacchione syndrome [29] there are also neurological abnormalities. Cleaver [21] was the first to describe excision repair defects, modelled on similar defects in bacteria. The worldwide frequency of the condition is about 1 : 250,000 [104]. There is a high rate of parental consanguinity.

Much has been learned about the condition by exposing cultured cells to UVL. If excision of a pyrimidine dimer is defective, irradiated cells will not be able to carry out unscheduled DNA synthesis (UDS) after irradiation. Tritiated thymidine may be used in the culture medium and when it is incorporated at any stage other than during the S phase of DNA replication, this is detected by a pattern of spots of incorporation in the autoradiogram, where repair has occurred. Most cases of XP show no such pattern [25].

Fussion of cells from related and unrelated XP subjects, and observation of the ability to carry out repair, has been the most revealing research technique. The nuclei of the fused cells do not fuse but a missing enzyme may pass between them if either possesses one that the other lacks. Studies of this sort have uncovered seven complementation groups (A-G) [3, 14, 23, 30, 53]. The question as to whether seven enzymes could be involved, an unexpectedly high number, or less, some or all derived from subunits, or non-enzymic cofactors, remains to be resolved. In addition to these seven groups there is a variant group with normal DNA repair, cells from individuals of which are sensitized to UVL damage by growth in caffeine after exposure [22]. All XP, A to G and variant cells have deficient ability to reactivate UVL irradiated adenovirus, double stranded DNA virus and purified DNA of SV40 [92].

Cleaver, [22] suggested that the variant XP may in fact have an abnormal polymerase, which has difficulty in replicating beyond damaged sites, leading to problems in both post-replication and excision repair. XP cells can repair single breaks caused by X-rays [55]; their ability to repair chemical injury varies with the type of injury [100].

Cleaver and Gruenert [24] have reported differences between different XP groups in the ability to repair psoralen adducts. They used angelicin (Ang) and 5-methyl-angelicin (5-MAng), which form DNA adducts when irradiated with UVA at 360 nm. Such adducts are excised rapidly from normal cells; 80–90% of the Ang adducts are excised in 24 hours, while 65% of the 5-MAng are excised in that time. XP-GpA cells excise about 20% of Ang adducts in 24 hours, XP-GpD about 55–60% and XP-GpE about 80%. The proportions of excision are about standard for GpsA and E but not for GpD. The GpD cells remove Ang and 5-MAng adducts much more efficiently than they remove pyrimidine dimers, suggesting that the normal enzyme which is deficient is more specifically oriented to the removal of dimers than to the removal of adducts. Ichihashi *et al.* [50] have observed a mild XP case classifiable as GpG but showing about 25% of normal UDS compared with less than 5% expected of other GpG cases. Thus there is evidence of heterogeneity at this gene locus.

As regards the neurological damage Robbins [102] has shown, using the ability to form colonies after various doses of irradiation, that neurologic damage correlates inversely with colony forming efficiency (CFE). It is suggested that chemical and UV type injury [98, 103] can have an effect in sensitive internal organs, e.g. the brain, and that the severest UV defects can be associated with neurological damage.

The relationship of XP type UVL sensitivity to the immune system is worthy of speculation and further research. Bridges [11] discussed a concept—the pseudopromoting effect of the immune system. There are many cells of the immune system to be found in the epidermis. The synthesis of immunoglobulins in the body is dependent on events broadly described as mutational, which include chromosomal rearrangement, and the theoretical possibility exists for an enhanced effect on immune function of sensitivity to UVL. Tohda and Oikawa [130] have scored sister chromatid exchanges (SCE) induced by UVL, and viability after UVL exposure, in lymphoblastoid lines derived from seven XP patients and six normals. UVL caused significant increases in SCE in both groups. In three XP lines which were deficient in UDS and sensitive to killing by UVL, very high frequencies of SCE were observed. One patient with De Sanctis-Cacchione syndrome had the highest rates of SCE and cell killing. Two out of four UDS proficient XP cell lines had normal levels of SCE but in two other UDS proficient XP lines from two XP patients with skin cancer, the frequencies of SCE were significantly above normal. Treatment of cells post-UVL with 1M caffeine enhanced SCE greatly in three of the four UDS proficient cell lines, with only a slight effect on the fourth.

Liao *et al.* [61] have reported on chromosomal aberrations in XP cells. Lymphoblastoid cells from two XP patients, two Cockayne syndrome (CS) patients and three normals were irradiated with 10 J/m² of 254 nm UVL. The

coromosome aberrations in the normals were close to zero, while in the XP and CS cells significant numbers of aberrations appeared, CS being the greater. Since CS patients have a normal rate of cancer, it may be concluded that chromosome aberrations do not per se contribute to cancer.

3.1.3 Ataxia Telangiectasia (AT)

This rare recessive disease has been a fertile source of research interest, second only to XP in its field. We have a great need to understand fully the effects of radiation on the body and XP and AT are two prototypical diseases to study in this context. In the skin there are many telangiectases, more prominent on sunlight exposed areas. Other signs are progressive ataxia, sino-pulmonary infections, a combined immuno-deficiency involving IgA, anergy of cutaneous responses to antigens, and *in vitro* lymphocyte unresponsiveness. Murphy and Zwerdling [71] have provided a succinct review. The most significant factor is an increased sensitivity to ionizing radiation comparable to the sensitivity of XP patients to UVL.

Much scientific effort has gone into efforts to correlate degenerative conditions and chromosome instability in a number of diseases. In AT, Shaham and Becker [112] have investigated a serum factor which they believe to underly a chromosome instability. They reported on a peptide of MW 500–1000. It was found in conditioned medium from AT skin fibroblast cultures, but not in extracts of cultured AT fibroblasts. Incubation of normal human lymphocytes stimulated with PHA in the presence of AT plasma or conditioned medium from AT fibroblast cultures caused increased breakage of lymphocyte chromosomes.

Many degenerative conditions have been investigated extensively for colony forming efficiency (CFE) of fibroblast lines derived from sufferers. Cellular senescence is generally believed to correlate with diminished CFE. Shiloh *et al.* [117, 118] found a significantly reduced CFE in AT fibroblasts compared with normal. The CFE of AT colonies is markedly reduced using some batches of newborn calf serum in the culture medium, while cell colonies from normal subjects are not batch sensitive. Fibroblast and epidermal growth factors but not insulin could correct the batch deficiencies.

Barfknecht and Little [5] studied the effects of alkylating agents on AT fibroblasts and found them to be hypersensitive. The test was based on CFEs of cells from three ATs and three normal subjects. The results were not conclusive. None of the three AT strains was abnormally sensitive to N-methyl-N-nitro-N-nitrosoguanidine. Two AT strains were sensitive to methyl methane sulphate, and all three were sensitive to ethyl methane sulphate. Two strains were sensitive to 4-nitroquinoline-1-oxide. Thus there was much heterogeneity of results, suggesting underlying biochemical variations in the ultimate AT genetic lesion.

Jaspers and Bootsma [51] investigated the rate of UDS induced by UVL in AT cultured fibroblasts, compared with normal. The AT cells were divided into excision deficient (ED) and excision proficient (EP) according to responses to ionizing radiations. The effect of X-ray exposure to AT-ED cells was to inhibit UVL UDS, while the effect on AT-EP cells was to increase UVL

UDS. This suggests different molecular defects in ED and EP cells. Painter [88] noted the greater radiosensitivity of AT cells but claimed that DNA synthesis was more resistant to ionizing radiation in these cells. To explain this paradox, he proposed that the delay in resynthesis in normal subjects allows greater time for repair of the lesion with more precise restoration of the original code. Doses of irradiation up to 20 Gy to AT cells caused a relatively small inhibition of DNA synthesis, which was almost totally due to inhibition of replicon initiation and not to inhibition of chain elongations as it is in normal cells.

Hariharan *et al.* [47] used the alkaline elution method to measure the repair of X-ray induced strand breaks in AT derived fibroblasts. With 2.5 Gy of X-rays, normal and AT fibroblasts repaired all single strand breaks in 30 to 60 minutes without significant differences. The pattern of rejoining was different at 12.5 Gy but again there was no difference between AT and normal. Thus it was concluded that single strand rejoining is not defective in AT.

Parshad *et al.* [90] using AT (N = 5) and normal control (N = 6) fibroblast cultures showed a much higher incidence of chromatid breaks and gaps following X-irradiation during the G2 phase of the cell cycle. They claim that the increased risk of cancer in AT homozygotes is shared by AT heterozygotes, who constitute about 1% of the population. They believe that this 1% have a repair deficiency in the G2 phase of the cell cycle, which could be monitored as an at-risk population. Mitchell *et al.* [67] have measured DNA polymerase levels in four normal donor cell lines and five AT cell lines. The mean polymerase levels were two-fold higher in the AT patients : thus deficiency of this enzyme cannot be the cause of the defect.

Other biochemical studies have been carried out. Smith and Paterson (1983) studied the effect of aphidicolin on *de novo* DNA synthesis, DNA repair and cytotoxicity in γ-irradiated fibroblasts, particularly in respect to AT [122]. Aphidicolin is an inhibitor of DNA polymerase and therefore of *de novo* synthesis of DNA. γ-irradiated normal and AT cells rejoined single strand breaks at normal rates with and without 5 mg/ml of aphidicolin. The delaying effect of aphidicolin on *de novo* DNA synthesis in AT made no difference to the number of strand breaks, suggesting that polymerase-α is not involved in the repair of the lesions resulting from γ-irradiation in AT or in normal subjects.

Shiloh and Becker [116] reported on the action of neocarzinostatin (NCS) *in vitro* on AT and normal fibroblasts. Low doses of NCS inhibited both the rate of replicon initiation and of chain elongation; there was more resistance to this inhibition in AT cells than in normals. Shiloh *et al.* [117, 118] reported on the sensitivity of AT fibroblasts to NCS measured by CFE in three healthy donors, eight homozygous AT and three heterozygous AT. The homozygous AT were significantly more sensitive to NCS killing than the controls, while the heterozygotes were intermediate. There was a direct correlation between NCS and ionizig radiation sensitivities. AT cells from a British patient were much more sensitive to both NCS and bleomycin than cells derived from North African and Middle Eastern patients.

In eight additional heterozygotes which also had intermediate values, inhibition of DNA synthesis was found to give results with NCS consistent

with the CFE results previously reported, for both homozygotes and heterozygotes [119]. Inhibition of DNA synthesis with NCS was reduced in AT compared with normal cells, but the inhibition resistant component of DNA synthesis typically observed following treatment with low doses of X-rays or bleomycin was not observed with NCS. DNA repair synthesis did not differentiate between AT homozygotes, AT heterozygotes and normals. This reduced inhibition of DNA synthesis rather than reduced extent of DNA repair synthesis correlates with the the sensitivity of AT homozygous cells. The hypersensitivity is mainly in respect of DNA breaking agents.

Shiloh *et al.* [120] studied the time course of strand breakage induction and repair in AT skin fibroblast strains and the effects of the quinone containing tumour antibiotics streptonigrin and adriamycin, and of hydrogen peroxide. The hypersensitivity response was accompanied by reduced inhibition of DNA synthesis compared to normal, after the use of these agents. The response of AT cells to paraquat, safranin A and ellipticin were not different from normal, suggesting that the strand breakage could be due to free radicals destroying the deoxyribose content of the chain. Bleomycin increased chromosome breakages significantly in AT homozygotes but not in heterozygotes [113].

3.1.4 Pigment variation—freckles, birthmarks and moles

In XP the name derives from the fact that the skin, where it is exposed to sunlight, is dry and scaly (xeroderma) and intensely freckled (pigmentosum) with light, dark-brown and black freckles. At an early age there is a likelihood of developing basal cell and squamous cell carcinomas, melanomas and various sorts of sarcomas in the exposed skin. There is no evidence of increased numbers of pigmented patches present from birth (birthmarks). In neurofibromatosis (NF) on the other hand, there are often an abnormally large number of birthmarks (*café au lait* patches). The young child with NF rapidly develops many freckles which, on exposed parts, fuse at an early age, while on covered parts they continue to appear, hence the diagnostic sign of axillary freckling [27].

Tuberous sclerosis (TS) on the other hand has depigmented patches similar in orientation and numbers to those of NF, and some subjects have large numbers of white freckles as well.

Nicholls [74] initiated studies into the various observable relationships between freckles, birthmarks, moles, neurofibromata and a number of other disorders which he included in a broadened classification of phacomatoses [75–83]. The underlying concept for all of these studies was one of somatic mutation occurring either spontaneously, or as a result of exposure to UVL (sunlight). A spontaneous change occurring before birth will cause a patch or birthmark, the size of which will be related to the stage of development of the embryo/foetus when it occurs. Correlations were found between most of the lesions studied, e.g. between the phenotype freckling and the occurrence of birthmarks and moles. Red hair was shown to be an autosomal recessive condition with respect to its effect on hair colour and autosomal dominant with respect to freckling, i.e. the heterozygotes are almost always freckled,

sometimes more noticeably freckled than the homozygotes. The phenotypic variations have not received much prominence, because in the areas of white residence in the Northern Hemisphere people do not develop a lot of freckles in childhood in many of the relatively sunless places, while in the tropics the dark skinned races have a low frequency of freckling genes and the dark pigmentation usually obscures any tendency to freckling. Sydney, Australia, has a large white population exposed to subtropical sunshine, and it was in Sydney that the studies were conducted.

Iris freckles were studied and these bear a statistical relationship to patches in the iris (iris sectors) or to heterochromia iridis, in which the two eyes are of different colours. Red haired Western European subjects have an average of 1.02 pigmented birthmarks, brown haired freckled subjects (heterozygous for red hair) have an average of 0.76 birthmarks and relatively unfreckled subjects have an average of 0.39 birthmarks (relatively unfreckled, because very few people in any population are without at least one freckle).

The relationship of moles and neurofibromata to their relevant freckling types is significant. The freckle is by definition and careful microscopic observation a monolayer of melanocytes exactly as is found in normal skin. A mole or neurofibroma is a multilayered neoplasm. There are always many fewer moles or neurofibromata than there are freckles. The mutational hypothesis can explain this in terms of numbers of mutations required to produce the lesion—the freckle or flat pigmented birthmark results from a single mutation in a gene involved in some way in pigment production: the production of the visible lession is due to a reproductive advantage of the mutant cell compared with those surrounding it. This reproductive advantage is justification for calling a pigmented birthmark (which is a large freckle) and a freckle, neoplastic. The interpretations of the mole, melanoma, neurofibroma and other such lesions is that, these are due to more than one mutation, giving rise to the neoplastic clone. For the simplest benign lesion the number of mutations may be small, but for example, in the case of the mole there may be several mutations affecting pigmentary status and a small number, perhaps only one or two, associated with the change in behaviour of the cells to produce the proliferating three-dimensional clone. Estimates of the number of mutations are not difficult to make, starting with the simplest and palest freckle or birthmark as a single mutation, and progressing through successive stages of darkening freckles to four or five levels of darker pigmentation. If the mutation theory is correct, the minimum number of mutable loci is three. The numbers of freckles at each level of colour change can be scored, and the scores of the darkest freckles can be compared with the scores of moles as a further estimator.

In the eye several shades of iris spot can also be seen in certain informative cases, and the eye colour variation, taking into account the spots, can be explained on the basis of two loci with four levels of colour. This is contrary to the often stated claim that the eye colour is due to a single locus with dominant and recessive alleles. The red hair gene has only a slight effect on eye colour, giving in the homozygote a reddish tinge to the eye pigment of a potentially brown-eyed person. The red hair locus has not been seen to have an effect in causing iris freckles.

Malignant melanoma may be due to 8 to 10 mutations in sequence in the mutant cell line, although this is the crudest of estimates, because almost all melanomas show some evidence of regression, believed to be due to an immune response, and this will inevitably affect estimates of mutation frequency. Moles also regress in time although usually quite slowly.

3.1.5 Neurofibromatosis (NF)

This dominantly inherited disease is one of the most common human disorders in its class, about 1 : 2,000 to 1 : 2,500. This figure is determined in surveys, as no clinic is ever likely to approach total ascertainment of cases in its area. Most cases only attend clinics rarely, if at all. The extreme manifestations of the condition are quite rare.

There have been very few generally accepted hypotheses to explain the joint occurrence of the *café au lait* patches, the freckles and the neurofibromata. Nicholls [79] suggested a hypothesis which he later [80] generalized to cover all of a wide range of phacomatoses (hereditary tumorous conditions with multiple small benign or malignant tumours) including retinoblastoma. Later Knudson [56] began publication of a series of papers which expressed the same idea as put forward by Nicholls, but which he limited in application greatly, never acknowledging the possibility that there could be many diseases following the rules originally laid down.

The hypothesis was, and it has now been given great support by the work of Cavenee *et al.* [16] using DNA methods in retinoblastoma, that the person who develops NF is a person who has inherited a mutant gene from one parent, which leads to the expression of a lesion in the skin or elsewhere, only if its normal allele also mutates. The mathematics of this is simple. With about 2,000 melanocytes per mm^2 of skin, if a specific mutation is going to occur at a rate of say $1 : 10^6$ per gene per annum, then more than a thousand such mutations can occur annually over the whole skin surface. The most obviously affected tissues in NF are the Schwann cells of nerve sheaths and the melanocytes, both types being derived originally from the neural crest. In the case of either cell, the mutation produces a reproductive advantage. Such a mutation in a melanocyte rapidly produces a pigmented spot. This mutation may be spontaneous, i.e. not induced by any known mutagen, and many birthmarks are often seen. Furthermore, covered areas of the skin of adults are usually densely freckled. These areas probably have had some UV irradiation, although one may discount this as the sole cause of the freckling. It is believed that the freckling tendency on UVL exposed areas rapidly leads the subject's skin to become one large freckle.

The neurofibromata (see also Section 3.1.4) according to the mutational hypothesis require not one but many mutations for their genesis. The clear evidence of regression in many neurofibromata make it difficult to hypothesize as to the number of mutations, but the necessary and sufficient number could be of the order of six, given the frequency of freckles and estimates of somatic mutation frequency.

It must be appreciated that freckling as discussed in the red hair phenotype and in NF is an epiphenomenon of mutation of single specific genes

while in XP, freckles and other lesions result from the vastly increased mutation rate affecting many gene loci due to the repair defect. Knudson's theory with respect to retinoblastoma was firmly locked in on lesions produced by just two mutations—contrary to the work of others [31, 84], who had hypothesized many mutations as a cause of cancer. Knudson's estimate of just two mutations for retinoblastoma must bear some element of doubt, because of clinical evidence of regression of these tumours in many cases.

Some of the modern techniques of study already quoted have been used to study NF. Schwenn *et al.* [109] reported that X-rays, UVL and an alkylating agent were all unable to show a difference in the stability of DNA between NF patients' cells and normal control cells. For a person who believes in the specific allelic mutation hypothesis, this is not surprising. Rowley and Kosciolek [107] have been searching for oncogenes as an aetiological factor in the neurofibromata without conclusive results.

One of the features of the NF syndrome is the not excessively rare occurrence of segmental NF. This fits the hypothesis of specific rather than general mutation, as the first mutation at the locus occurring in somatic cells only creates a field in which the second mutation can cause lesions. Pullara *et al.* [97] have recently added another case to the list of reports of segmental NF.

3.1.6 Tuberous sclerosis (TS)

This condition is rarer than neurofibromatosis (about 1 : 20,000 in most populations): those sufferers who are retarded, which is a high percentage, are frequently severely retarded with no prospect of reproduction. It is not surprising therefore that this dominantly inherited condition is found to have a high mutation rate for the maintenance of its population frequency. There are a number of internal and external effects of the disease : internally there are various tumours, externally there are depigmented patches, shagreen patches, white freckles on some sunlight exposed areas, facial angiofibromas showing a probable involvement of UVL in their etiology, and small fibrous tumours in the skin, subungually and in the gingivae.

TS was included by Nicholls [80] amongst those numerous diseases which he regarded as having a common aetiological mechanism : mutation at one gene of a locus, with development of lesions if the second gene for that locus should also mutate. These mutant genes were perceived as being totally inactive, despite the appellation dominant, and could well be small chromosomal deletions. So far there is no evidence for this, except in retinoblastoma and Wilms' tumour but as the DNA technology applied to medical genetics progresses, proof should be forthcoming if this is the mechanism.

As with NF and the red hair phenotype, the occurrence of birth-marks is hypothesized to be due to mutation occurring in a melanocyte in the embryo or foetus with growth of a clone of melanocytes changed by the mutation. The change postulated must be highly specific; a protein product important to the function of the cells is no longer being produced in those cells. There has been some speculation as to what type of protein might be involved. It would have to have widespread functions in the body with effects in the brain, heart,

kidneys, skin and other organs. It would need to be involved with melanocyte function and would probably be a change brought about by mutation within the melanocyte itself, as a result of UVL to cause white freckles. The melanocytes of the depigmented spots and patches of TS have few melanosomes and those that they have are small and produce little pigment [38].

The central nervous system in TS can be affected in three principal ways—retardation, epilepsy and the tubers, which are tumorous or poorly developed parts of the brain (hamartomas). There are inhibitory neurotransmitters in the brain and the absence of a receptor for such a neurotransmitter in the lesions might lead to the combined effects observed in the disease. If the mutational theory is correct, it could be supposed that the depigmented areas of the skin might have counterparts in the brain, where the developmental process has deviated from the normal as a result of mutation.

3.1.7 Malignant melanoma

As a cancer, malignant melanoma has a strange fascination—the sufferers are often relatively young, the cells of origin are well defined, the biochemistry of the cells has been studied extensively and the spread of the cancer is often devastating. A familial tendency has been noticed since the earliest studies, and the existence of a melanoma gene has been postulated recently [44]. Dysplastic nevi, which they have found in dominantly transmitting melanoma families, frequently have some of the features of rapidly growing nevi with areas of regression [82]. The familial pattern can come about by the coincidence of a genetic tendency to moles allied to a freckling phenotype seen in subjects heterozygous or homozygous for red hair genes [78], indiscrete exposure to excessive sunlight for geographic, social or occupational reasons, and the possibility of an unusual mutability of DNA.

Rapidly growing dark-brown or black, benign dysplastic nevi are already neoplastic in the original sense of the word. If multiple mutations are needed to produce a cancer, the number of mutations may not be great in the case of malignant melanoma and the malignant transformation may occur quite rapidly (see also section 3.1.4). The production of increased amounts of melanin in the dark-brown or black nevus should be protective against UVL mutagenesis, but it can be calculated that if UVL causes a hundred-fold increase in mutation rate, an initial lesion will continue to undergo further mutations in some of its cells spontaneously, once the numbers of cells increase by say a few thousand or million. Thus the malignant transformation can be readily postulated when cell numbers in the nevus reach a certain size. There is clinical evidence that our immune system is very active in protecting us from certain death from such lesions, if they occur.

The study of melanoma has been greatly enhanced by the existence of many mouse melanomas, which have been passaged from mouse to mouse and/or cultured for many years. Genetic variation in subcultures can be seen [124]. These variations underly the different abilities of different mouse B16 melanoma subcultures to metastasize in different individuals of the same strain of mouse.

Ploidy variations are common in human melanomas, Sondergard *et al.*

[123] have reported on melanomas ($n = 35$), metastases ($n = 20$) and nevi ($n = 16$). Significantly, while 76% of the primary cancers had ploidy variations, 25% of the benign nevi also had clones with abnormal DNA content. Ten primary melanomas, two metastases and one nevus each had three or more clones. Heteroploidy therefore was not absolutely correlated with malignancy, but it did correlate with marked cellular anaplasia and a bad prognosis.

Shtromas *et al.* [121] have also investigated clonality and suggest the possibility of DNA amplification as a correlate of increased malignancy. The cell line they used contained hypodiploid and hypotetraploid cells; one subline had some cells with homogeneously staining regions (HSRs), while another did not. These sublines were injected into nude mice and the growing cells were eventually separated by flow cytofluorimetry. The subline containing cells with HSRs was the most aggressive, and during growth in the nude mice, the hypodiploid cells from this line increased their frequency of HSRs from 40 to 90%.

Because melanoma in man is often incurable, and rarely responds well to radiation, much work has been done on cultured cells and in mice on the activity of antitumour compounds against the DNA of the tumours. The mouse B16 model has been used for testing platinum compounds, cisplatin and hydroxymalonato-diammine platinum II (Pt[OH Mal $(NH_3)_2$]). Pera *et al.* [95] assessed the ability of these compounds to bind to DNA of the melanoma. Selective killing of B16 cells was achieved with Pt[OH Mal $(NH_3)_2$], but cisplatin was relatively unselective. The greater selectivity correlated with greater amounts of platinum bound to the DNA. No significant excision of total DNA bound platinum from the tumour was observed for either drug up to 48 hours after administration.

Drug studies on human cell lines have also been revealing of fundamental characteristics of the tumour DNA. Parsons *et al.* [91] examined a melanoma line, which had developed maximal resistance to melphalan. This resistant line (MM253-12M) was 2.6 × Do, 1.5 × size, 1.3 × RNA, 1.4 × protein and 2.6 × DNA compared with the sensitive parental cell line. There was no evidence of detoxification of melphalan in the resistant line; melphalan transport was the same in the two lines, and so was the total melphalan uptake, giving rise to a reduced level of binding in the case of the sensitive line. The degree of DNA cross linking in MM253-12M was 50% less than in the parental line MM253. The conclusion was that the increased resistance of MM253-12M resulted from the decreased cross linking. Hansson *et al.* [46] obtained similar results with melphalan and melanoma cells.

Wurm *et al.* [140] extracted RNA from lymphoid organs of guinea pigs, which had been injected with fish melanoma and claimed that this RNA was protective of other melanoma prone xiphophorin fish. Such findings are exciting if they can be reliably reproduced. Aarons *et al.* [1] injected B16 melanoma into the hind limb of C57 B1/6J mice and allowed the cells to grow until the ninth day when the limb was amputated. Immune RNA to the B16 melanomas prepared from the spleens of immunized sheep significantly improved survival of the mice and retarded the spread of metastases.

3.1.8 Premalignant and malignant epithelial tumours of the skin

Skin cancer in ancestral human populations is not common. The original European populations were stratified according to skin colour with the fairest in the highest latitudes and the ones with darker skins in the Mediterranean region. Skin cancer in such populations was not a common disease unless they migrated to a country such as Australia. In Australia some stratification according to latitude has also occurred, but to nothing like the extent of the ancestral populations—fair-skinned Irish and Scottish can be found in the north and Greeks and Italians are very numerous in Melbourne and other southern cities where the UVL levels are not such a problem. Thus in Northern Australia, skin cancer is an exceedingly common disease and the public and the medical profession are so aware of it that most such cancers are rapidly removed. There is no doubt as to the negative correlation between the frequency of the disease and the depth of skin pigmentation. The same applies to melanoma. Whether other genes besides skin pigmentation genes are important in the resistance to UVL carcinogenesis is not known, except in the extreme case of XP. It might be supposed that populations such as the Irish who frequently have very fair skins with high numbers of red hair homozygotes and heterozygotes would also have other genes giving susceptibility to skin cancer. The evidence, such as it is, does not point this way. XP has been reported more often in Egyptian and Middle Eastern people than in people of Northern Europe. This may be an accident of ascertainment or of founder effect, but it does not point towards a high lethality of this gene for UVL sensitivity in lands with much sunshine.

There has not been a lot of basic scientific investigation of skin cancers except for melanomas. Newton *et al.* [73] investigated aneuploidy in Bowen's disease, which is a premalignant condition, using flow cytometry to demonstrate aneuploid clones as well as euploid cells in lesions from six patients. Aneuploid clones were found in all six lesions, a finding which is consistent with other similar investigations, for example, of melanoma. Holm [48] found aneuploidy in 75% of squamous cell carcinomas of the head and neck.

3.1.9 Multiple basal cell carcinoma syndrome (MBCCS)

In this dominantly inherited condition, the carcinomas develop, in young adults, more commonly in sunlight exposed areas of the body, especially the face. Some of the lesions evolve slowly while others go on rapidly to ulceration [17]. Some of the lesions are focal as are the skin lesions, some are not, and this disorder could well be due to chromosomal deletions varying in size in different cases.

Efforts to define specific differences from normal in the cells of MBCCS, such as differential effects of various agents on DNA, have not been consistently successful. Ringborg *et al.* [101] reported a 25% diminution in repair synthesis in seven patients with MBCCS compared with 39 healthy subjects. Chan and Little [17] reported that diploid fibroblasts from MBCCS patients were hypersensitive to the killing effects of ionizing radiation. The cultured fibroblasts of five patients compared with six controls gave Do values of 0.98 Gy for the patients and 1.42 Gy for the controls, results which must be

compared with a value of 0.44 Gy for ataxia telangiectasia (AT), a disease which is generally believed to be due to sensitivity to ionizing radiation. Don *et al.* [32] report an experiment using fibroblasts from the two sides of a patient, who had MBCCS on his left upper trunk but not on his right. Those from the left side had 45% less ability to metabolize benzo-a-pyrene than those from the right.

Shelley *et al.* [115] reported a patient with a quadrant sector of the body affected by the condition. Camisa *et al.* [15] had a unilateral case and Nicholls (unpubl.) has observed such a case also. These segmental cases support the general proposition of a mutation, which can be germinal or somatic, at one allelic site of the locus with a second mutation of the other allele to act, either as a cause of the neoplasm or, at least and more likely, as the initiator of change which eventually expresses itself as cancer.

It is accepted that the first appearance of tumours in the MBCCS is on sunlight-exposed areas of the body, implicating UVL as an initiator of change in the basal cells of the epidermis. Allison [2] reported carcinomas in MBCCS patients, who had been given ionizing radiation 20 years earlier in the affected areas. That two different types of radiation with different repair mechanisms can cause the same type of lesion supports the idea of a biochemical change quite specific to the basal cell, which can be induced by any mutagenic mechanism.

3.1.10 Cutaneous T-cell lymphoma (CTCL)

Edelson [33] has reviewed the recent work on lymphoma of the skin, including the introduction of a method of scoring T-cells by their receptor for sheep erythrocytes, and the recognition that Sezary syndrome, mycosis fungoides, leukaemias with widespread skin infiltration and lymphomatoid papulosis are all T-cell malignant or pre-malignant conditions. Most T-cell malignancies in adults were usually present as cutaneous disorders, and the cells usually belonged to the helper cell class. With the availability of monoclonal antibodies, it was revealed that CTCL is a malignancy of OKT-4 positive helper cells. It was realized that affinity for the epidermis was usually the less malignant stage, but that, in time, the disease progressed to a more aggressive, less cutaneously oriented stage. The clonal origin of the lymphomas suggested primary origin in the skin and the possible involvement of UVL in the etiology.

Ralfkiaer *et al.* [99] have looked at the DNA content of lymphocytic cells infiltrating the skin in normal patch tests and in CTCL. Fifteen of 18 and 10 of 11 respectively showed similar patterns of hyperdiploid cells. They state that this indicates that the presence of hyperdiploid cells does not necessarily infer the presence of mutant clones within the skin. Their observations invite caution in interpretation, particularly where the presence of clones might be considered an indicator of malignancy. A patient reported with XYY chromosome constitution and mycosis fungoides [86]. An IL-2 dependent T-cell line was established from blood mononuclear cells: all cells of this line had the extra Y chromosome.

Berger *et al.* [7] investigated 13 CTCL patients by restriction endonuclease

digestion of peripheral blood lymphocytes using EcoRI, BamHI, BglII and Hind III. All 13 patients had a clonal rearrangement of the JH region in the T-cells. There was a higher percentage of bigenotypic clonality in these CTCL cases than in other T-cell neoplasms. DNA hybridization was suggested as a regular procedure for the diagnosis of clonal malignancies.

4. Comments and Conclusion

In this chapter, no effort has been made to cover all genetically determined disorders of the skin, nor to provide a complete clinical description of those covered. Up to a few years ago, the cellular genetics of the skin was mainly observed by light and electron microscopy with some further studies at the level of biochemistry and histochemistry. The advent of DNA technology in this field, as in many others, is creating a slow but certain revolution, and the main objective here is to highlight some aspects of the already massive literature, which has heralded that revolution. In the space available, it is not possible to be totally comprehensive in any direction, but it is hoped that the reader will recognize some of the important trends and be able to follow up on some of the most significant authorships.

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5

Monitoring Human Exposure to Carcinogens by DNA Adduct Measurement

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1. Introduction

There are now several techniques available for the sensitive detection and quantitation of carcinogen-DNA adducts in human populations. Measurement of DNA binding is considered a relevant dosimeter of human exposure, since it is well established that the initiating step in carcinogenesis is DNA binding [24]. While studies on DNA adduct formation in animals have used radiolabelled carcinogens, new methods had to be developed to measure the low levels of adducts that were expected in humans. One technique utilizes specific monoclonal or polyclonal antibodies to particular DNA adducts (for review see [34, 53]). Antibodies can be made with two different types of immunogens. The *in vitro* modified DNA can be complexed with methylated bovine serum albumin before mixing with adjuvant. Alternatively, the ribose form of the isolated adduct can be coupled to protein with periodate [7] before mixing with adjuvant. These antibodies can be used in highly sensitive immunoassays to quantitate adducts levels. They can also be used in immunohistochemical studies to localize adducts in specific tissues. Antibodies have been developed against DNA modified by benzo (a) pyrene [36, 46, 49], acetylaminofluorene [2, 21, 37, 42], platinum [10, 23, 35], aminopyrene [19], 8-methoxypsoralen [43, 63], aflatoxin [17, 18] and melphalan [54]. In addition, addition, antibodies have been made to several alkylated based [26, 27, 56, 58] as well as radiation damaged DNA [22, 52, 57]. The other technique used to monitor levels of adducts in humans is the ^{32}P postlabelling technique, discussed in detail here.

2. Antibodies to DNA Modified by Benzo (a) Pyrene Diol Epoxide I

Benzo (a) pyrene, a carcinogenic polycyclic aromatic hydrocarbon, is a ubiquitous environmental pollutant found in mainstream and sidestream cigarette smoke, urban air and smoked and charcoal broiled foods. To develop techniques to monitor human exposure to BP, monoclonal antibodies were made against DNA modified by benzo(a)pyrene diol epoxide (BPDE-I), the metabolite of BP responsible for the majority of DNA adduct formation [46]. Antibody 8E11 was developed from the spleen cells of animals immunized with BPDE-I-guanosine coupled to KLH, while antibody 5D11 was developed from animals immunized with BPDE-I-DNA. Antibodies were characterized by competitive enzyme linked immunosorbent assay (ELISA). The general protocol for ELISA is given in Fig. 1. Ninety-six microwell plates were coated with the antigen, DNA modified *in vitro* with BPDE-I, by drying phosphate buffered saline solutions in the wells. The washed plates were then blocked by incubation with 1% foetal calf solution (FCS) to prevent nonspecific binding to the plate. A competitive mixture of diluted antibody and standard amounts of BPDE-I-DNA or unknown samples was then added to the wells. After incubation and washing, the second antibody coupled to an enzyme (goat antimouse IgG-alkaline phosphatase) was added. Finally, the substrate (p-nitrophenylphosphate) was added. Colour development is directly related to antibody concentration. As with all competitive assays, higher antibody in the well means less antigen in the test sample. A typical standard

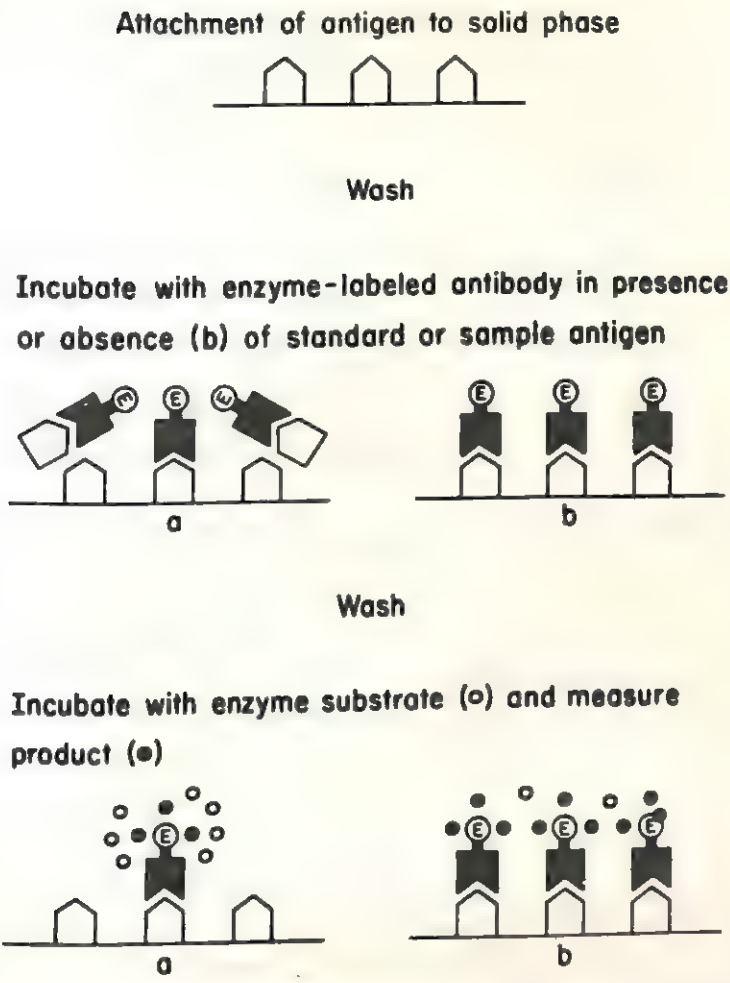


Fig. 1: Schematic protocol of the competitive enzyme linked immunosorbent assay (ELISA)

curve is shown in Fig. 2. Higher antigen levels give higher inhibitions of antibody binding. A simple measure of antibody affinity for a particular antigen is the amount giving 50% inhibition in a competitive ELISA. Table I shows that the highest reactivity of antibody 8E11 was with BPDE-I-dG, the deoxyribose form of the original immunogen (50% inhibition at 145 fmole). Relatively similar crossreactivities were seen with the modified DNA (50% inhibition at 350 fmole) as well as the free tetraols (50% inhibition at 250). This antibody thus recognizes the BP chromophore itself as well as the adduct. Antibody 5D11 also recognizes the antigen it was made against with the best sensitivity (50% inhibition with BPDE-I-DNA at 19 fmole). Lower crossreactivity was seen with the monoadduct isolated from DNA (50% inhibition at 21000 fmole) and no crossreactivity was seen with the free tetraols. This antibody thus recognizes the adduct and some of the surrounding DNA structure. Both antibodies have lower reactivity with DNA modified by BPDE-II, a stereoisomer of BPDE-I. Neither antibody 8E11 or 5D11 reacts

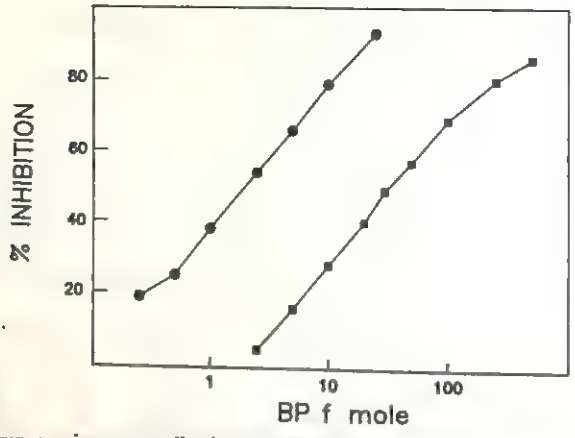


Fig. 2: Standard curve using an antibody to BPDE-I-DNA with fluorescence (●) or colour (■) endpoint detection

Table 1: Competitive inhibition of monoclonal antibody binding to BPDE-I-DNA fmole causing 50% inhibition

	8E11	5D11
BPDE-I-DNA	350	19
BPDE-I-dG	145	21000
BPDE-I-tetraols	250	$>10^5$
BPDE-II-DNA	$>3 \times 10^4$	4400
BPDE-II-dG	$>10^5$	$>10^5$
AAF-DNA	$>10^5$	$>10^5$
AP-DNA	$>1.5 \times 10^4$	$>1.5 \times 10^4$

with DNA modified by two other carcinogens, acetylaminofluorene (AAF) or aminopyrene (AP). Both of these carcinogens form adducts at the C8 position of guanine in contrast to BP which binds to the N2 position of guanine. More recently, we have tested DNA's modified by several other aromatic hydrocarbon diol epoxides [44]. Similar or 10 to 100-fold lower crossreactivity was seen with DNA modified by two different benz(a)anthracene and one chrysene diol epoxides. These epoxides all have similar stereochemistry as the BPDE-I adduct. Crossreactivites were also seen with a polyclonal antibody made against BPDE-I-DNA in collaboration with Dr. M. Poirier, NIH [36]. For this reason, DNA adducts levels are expressed as BPDE-I-DNA antigenicity and may result from multiple diol epoxide adducts. It is expressed as fmole equivalents of BPDE-I-dG, which would give similar inhibition in a competitive ELISA.

These antibodies can be used to measure adduct formation in animals treated with BP as well as humans with occupational or environmental exposure. This is also done by competitive ELISA. Sensitivities can be increased by carrying out the assay as in Fig. 1 but with the use of substrates that become fluorescent after phosphate hydrolysis. With alkaline phosphatase conjugates the substrate normally used is methyl umbelliferyl phosphate. Sensitivities can be ten-fold higher with fluorescence detection (Fig. 2).

With our most sensitive antibody and fluorescence detection, as little as 1 fmole of adduct can be measured. Since up to 50 μ g of DNA can be added to a microwell, the absolute sensitivity of the assay is one adduct in 10^8 nucleotides. Even higher assay sensitivities can theoretically be obtained with an antibody to a monoadduct. In this case, the DNA can be digested to mononucleosides and the adduct separated by some chromatographic procedure before quantitation in an immunoassay. Large amounts of DNA can be used since only the adducts are measured, thus increasing the sensitivity.

A number of different animal samples have been assayed by ELISA for BP adducts including those exposed to coal tar [25] as well as BP itself [31]. Human exposure has also been measured. Tissue samples include white blood cells, placenta and lung. Studies on cigarette smokers and nonsmokers indicated that placental DNA adducts were present in both populations [9]. While adduct levels were higher in smokers compared to nonsmokers, this difference was not significant. We have recently measured adducts in lymphocyte DNA of smokers and nonsmokers [32]. The number of samples with detectable adducts is much lower for lymphocyte DNA compared to placental DNA and did not differ in smokers and nonsmokers. Among positive samples however, smokers had higher adduct levels than nonsmoker. Studies on BP adducts are complicated by the ubiquitous nature of this environmental contaminant making control unexposed populations hard to identify. Nonsmokers may be exposed to significant amounts of BP through environmental or dietary exposure.

A number of studies have been carried out monitoring occupational exposure to BP. We have measured lymphocyte adducts in foundry workers in Finland in collaboration with Dr. K. Hemminki. Workers were classified as having high, medium or low exposure to BP based on their job description. Adducts were detectable in all workers with medium and high exposure. Mean adduct levels increased with increasing exposure (Table 2). Another group has used the same antibody to measure lymphocyte adduct levels in foundry workers [47] and coke-oven workers [15, 16]. In addition, antibodies to the adducts were seen in the sera of a number of workers [16].

Table 2. Foundry worker exposure to BP and lymphocyte DNA adduct levels

	Exposure level μgBP/m^3	Mean adduct level fmole/μg
Control group (n = 10)		0.036
Low exposure (n = 18)	<0.05	0.144
Medium exposure (n = 13)	0.05–0.2	0.372
High exposure (n = 14)	>0.2	0.900

3. Antibodies to DNA Modified by 8-Methoxypsoralen and UVA Light

8-Methoxypsoralen (8-MOP) plus ultraviolet A light (UVA 320–400 nm), PUVA, is used clinically in the treatment of psoriasis, a hyperproliferative disease of the epidermis [30] and extracorporeally as a cytoreductive treatment

for the leukaemic phase of cutaneous T cell lymphoma (CTCL) [6]. When photoactivated, 8-MOP reacts with pyrimidine bases, primarily thymine. Two different types of adducts are formed, monoaddition products and cross-links [4, 50]. *In vitro* studies have shown that these photoadducts are mutagenic [29, 48, 60] and can induce chromosome damage [3, 20, 28]. *In vivo* studies have demonstrated that 8-MOP plus UVA induces squamous and basal cell carcinoma [61, 62]. A recent follow up study of PUVA patients reported that exposure to high doses over seven years increases the risk of cutaneous squamous cell carcinoma ten-fold [51]. Patients treated clinically with PUVA thus provide an excellent population for the development and validation of DNA adduct monitoring techniques. Patients are exposed to high, well-defined doses and control populations are easy to identify.

We have developed monoclonal antibodies that specifically recognize PUVA modified DNA [43]. These antibodies do not react with nonmodified DNA or free 8-MOP. In a competitive ELISA, 50% inhibition with 8-MOP-DNA occurs at 17 fmole with antibody 8G1. This can be lowered to about 4 fmole with fluorescence detection of enzyme activity in the ELISA. Since 8-MOP forms both monoadducts and a crosslinked adduct, synthetic polymers were used to better characterize the antibody. Poly(dA-dT)-poly(dA-dT) can form both monoadducts and crosslinked adduct while poly(dA)-poly(dT) can form only monoadducts. Crossreactivity of the antibody with both these modified polymers (Fig. 3) indicates that antibody 8G1 preferentially recognizes monoadducts. Better sensitivity is seen with the homopolymer (50% inhibition at 13 fmole) compared to the alternating polymer (50% inhibition at 77 fmole). This was confirmed by testing the antibody with fractions isolated by HPLC from an enzyme digest of 8-MOP poly (dA-dT)-poly(dA-dT) [43]. Highest reactivity was with those fractions containing the 4 5 monoadduct. Crossreactivity of the antibody with two other psoralen modified DNAs was also tested. Significant reactivity was seen with 4-aminomethyl-4, 5, 8-trimethylpsoralen modified DNA (50% inhibition at 580 fmole) and dimethylangelicin modified DNA (50% inhibition at 104 fmole). In contrast, no crossreactivity was detected with two other carcinogen

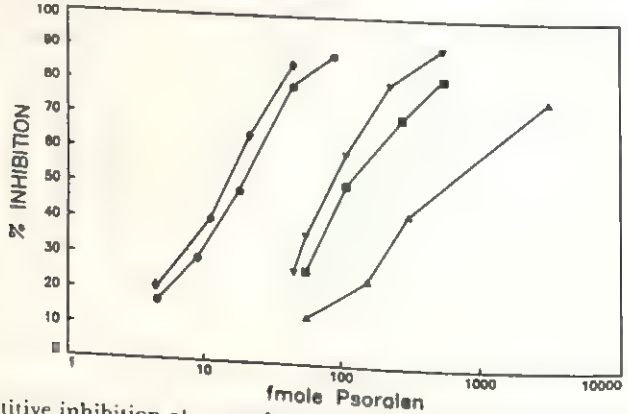


Fig. 3: Competitive inhibition of monoclonal antibody 8G1 binding to 8-MOP-DNA. The competitors were 8-MOP-DNA (●), 8-MOP-poly (dA-dT)-poly(dA-dT) (▼), 8-MOP-poly(dA)-poly(dT) (◆), DNA-DNA (■) and AMT-DNA (▲).

modified DNAs, BPDE-I-DNA or acetylaminofluorene modified DNA. Similar to the results seen with the antibodies to BPDE-I—DNA, significant crossreactivity occurs with DNA adducts having structural similarity to the original antigen.

We have used this antibody to monitor adduct levels in circulating lymphocytes of CTCL patients. Detectable levels were seen in all patients tested [45]. They were in the range of one adduct/10^6 nucleotides. High levels were expected, since, in this treatment, lymphocytes are irradiated *ex vivo*. In contrast, no adducts could be detected in the lymphocytes of psoriasis patients treated by skin irradiation. Levels were thus below one adduct/10^8 nucleotides, the limit of sensitivity of the ELISA.

The antibodies to 8-MOP-DNA have also been used in immunoflurescence studies to localize adducts in tissue samples (59). Human keratinocytes have been treated with 8-MOP and UVA and stained with adduct specific antibody followed by fluorescein-conjugated anti-mouse-IgG antibodies. Fixed cells are first treated with RNase to eliminate antibody binding to RNA adducts and with proteinase K to make the DNA more accessible to antibody binding by removal of DNA binding proteins. Finally, the DNA is denatured *in situ* with acid to make the adduct more accessible to the antibody. Antibodies generally have higher reactivity with denatured, single stranded DNA than with double stranded DNA. Specific nuclear staining was seen after treatment with 0.25–10 μg/ml of 8-MOP and 12 J/m^2 UVA. At high doses, nuclear staining was homogeneous, while with doses below 0.5 μg/ml staining was granular and weak (Fig. 4). No staining of the cytoplasm could be seen. Dimethyl sulphoxide treated control cells had no specific staining (Fig. 4), nor did other controls including use of a nonspecific antibody and treat-

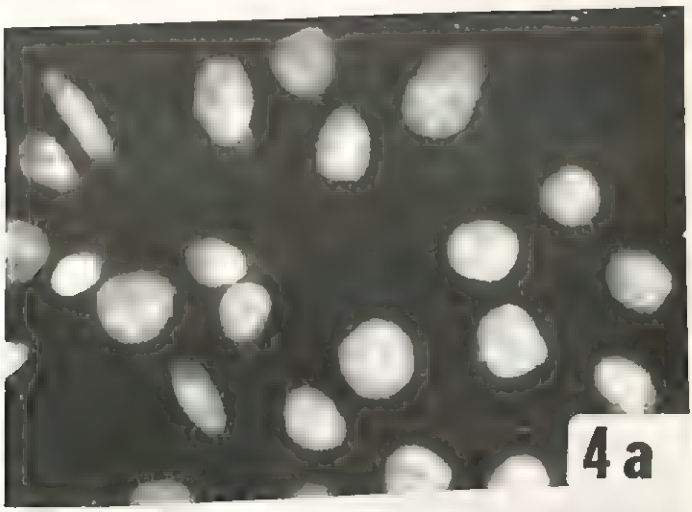
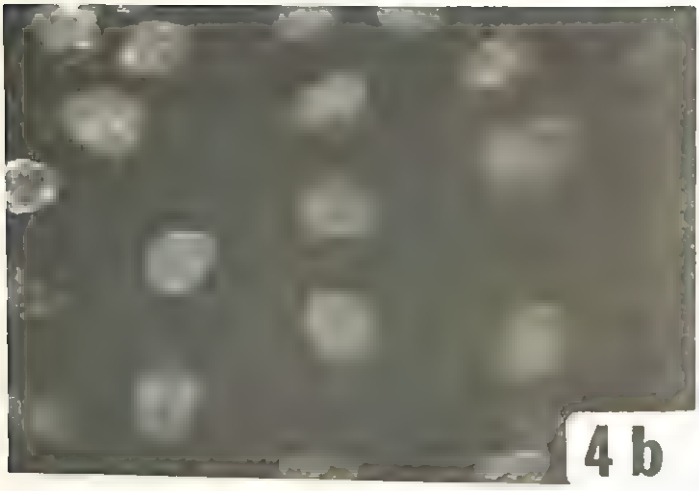


Fig. 4: Immunofluorescence staining of primary human keratinocytes treated with various concentrations of 8-MOP or untreated control. Anti 8-MOP-DNA antibody was applied and slides then incubated using fluorescein isothiocyanate-conjugated goat anti-mouse IgG. A, 10 μg/ml 8-MOP treatment; B, 0.25 μg/ml; C, dimethylsulphoxide treated control; D, immunofluorescence staining of mouse skin from an animal treated with 30 μg 8-MOP/g body weight. All samples received 12J/cm^2 UVA.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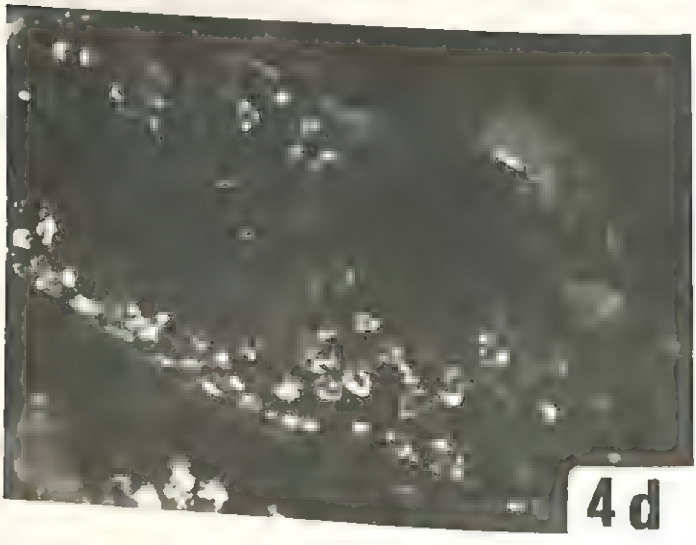
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ment with DNase before staining (not shown). DNA was also isolated from the keratinocytes and adducts measured with the competitive colour ELISA (50% inhibition at 17 fmole) or the fluorescence ELISA (50% inhibition at 4 fmole). This allowed determination of the sensitivity of the immunofluorescence technique in terms of adduct levels detectable. The lowest treatment level of keratinocytes that gave positive immunofluorescence staining was 0.25 μg/ml 8-MOP. Adduct levels for this sample were 9.0 fmole/μg DNA corresponding to 2.9 adducts/10^6. Thus the immunofluorescence technique is not as sensitive as the quantitative competitive ELISA with either colour or fluorescence detection. Higher sensitivities have been reported with the use of video-enhanced computer-assisted microscopy. As little as several hundred adducts per cell (seven adducts/10^8 nucleotides) have given positive results with a high affinity antibody to the O^6 methyl guanine adduct [1].

Animals were also treated with PUVA and sections stained. Positive staining was localized mainly in kerationocytes of the epidermal layer (Fig. 4). A control animal receiving UVA without 8-MOP had not positive staining (not shown). We have now begun to use this technique to monitor adduct levels in the skin of psoriasis patients treated with PUVA. It is hoped that these data will allow the determination of adduct levels required for therapeutic effect. This may alloy adjustment of 8-MOP and UVA dose to maximize clinical response and minimize potential risk for cancer development.

4. Antibodies to Other Carcinogen-DNA Adducts

Several groups are involved in the development and use of immunologic techniques to monitor human carcinogen-DNA adducts. Antibodies to the DNA adducts formed by cis-diamine-dichloroplatium are being used to monitor lymphocyte DNA adduct levels in patients treated with this cancer chemotherapeutic agent. Large individual differences in adduct levels have been seen and efficacy of treatment seems to correlate with formation of detectable adducts [11, 41]. Alkylated DNA adducts have been measured in tissue samples with antibodies to O^6 methyl guanine [55]. Large amounts of DNA were available, so sensitivities could be increased by enzymatic digestion of the DNA followed by separation of the adduct by HPLC before immonoassay. Exposure to aflatoxin has been measured in a Chinese population at high risk for liver cancer with an antibody which recognizes the chemically unstable N7 adduct [12]. This adduct is excreted in urine and can be isolated with an affinity column.

5. Postlabelling of Carcinogen-DNA Adducts

Another approach developed to quantitate carcinogen-DNA adducts enzymatically incorporates radiolabel in the DNA constituents. First the DNA is digested enzymatically with micrococcal endonuclease and spleen exonuclease to deoxyribonucleoside 3′-monophosphates of normal and adducted nucleotides. This is followed by labelling of the 5′ position with [^{32}P] ATP and polynucleotide kinase to produce deoxyribonucleoside 3′ 5′ biphosphates. Thin layer chromatography on PEI cellulose separates the labelled

normal nucleotides from the adducted ones. Further chromatography in solvents containing urea allow fingerprinting of the adducted nucleotides. Samples are detected by autoradiography and can be quantitated by Cerenkov counting. Originally developed by Randerath and Gupta [13, 14, 38, 39], we have recently begun to use it to monitor adduct levels in human DNA samples. In contrast to the antibody approach which requires the development of specific antibodies to each adduct of interest, the postlabelling technique has the potential ability to measure multiple adducts. In addition, the carcinogen responsible for adduct formation does not have to be known. This does make absolute quantitation difficult, since different adducts can be labelled with different efficiencies. The efficiency of labelling of a new adduct would be unknown.

About 80 adducts have been detected with this technique, including those resulting from aromatic hydrocarbons, aromatic amines, estrogens and methylating agents. Sensitivities of one adduct in 10^7–10^8 normal nucleotides are readily obtainable with hydrophobic adducts. Using a new procedure to enrich the sample in adducts, sensitivities as high as one in 10^9–10^{10} have been reported [40]. This procedure further digests the DNA with nuclease PI, which selectively removes phosphate from the normal nucleotides. The adducted nucleotides are resistant to digestion. Since the kinase will only label a monophosphate, the normal nucleotides are not labelled. Adducts which result from simple alkylations can also be detected but not with the same sensitivity, since the thin layer chromatography system does not allow as good a separation from the normal nucleotide bisphosphates as with the hydrophobic residues.

An adduct has been detected in placental DNA of smokers but not in nonsmokers [9]. Unfortunately, the identity of the adduct is unknown but it did not seem to correspond to those produced from several aromatic genotoxic chemicals present in cigarette smoke including BP. A smililar spot was seen in mouse skin after treatment with cigarett smoke condensate and may help in the identification of the adduct [8]. Other studies have detected adducts in exfoliated mucosal cells [5] and in human bone marrow mononuclear cells [33] of smokers and nonsmokers and several other exposures (e.g. betel-nut chewers). Similar adducts were seen in both exposed and unexposed samples.

We have begun to look at lymphocyte DNA samples from coke-oven workers and controls. Several adducts have been detected in samples but as with the smoker and nonsmokers data, the identity of the adducts are unknown (Fig. 5). These studies are ongoing and being extended to other exposure groups.

6. Summary

Two techniques are now available for the detection of carcinogen-DNA adducts in human samples. Both immunoassays and the postlabelling technique have sufficient sensitivity to measure the levels of adducts seen with environmental exposure. These assays can serve as tools in the study of

Fig. 5: 32P postlabeling of lymphocyte DNA from coke oven workers.

occupational or environmental carcinogenesis. They measure the biologically effective dose of carcinogens, the amount that actually reaches the target DNA. They can serve as molecular dosimeters, markers of exposure. This use has already been validated by the number of human studies carried out to date. Another important use would be as potential markers of risk and an aid in risk assessment. For example, comparable DNA adduct dosimetry data in a human population with unknown cancer risk and in experimental animals with known tumour incidence may help in extrapolation from animals to humans. There are several potential problems in this approach. The markers discussed here are related to DNA adduct formation and thus measure early or initiating events in the carcinogenic process. They do not take into account promotion or progression and may therefore be only partial predictors of risk. Background levels of adducts must also be determined as well as persistence. In addition, most studies have measured DNA adduct levels in lymphocyte DNA, since blood is readily and repeatedly available. However, the target tissue is different (e.g. liver, lung) and only an indirect measure of the biologically effective dose may be obtained. Future stuies as well as the development of techniques to monitor human exposure to promoters will provide additional information about the usefulness of these assay in risk assessment.

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7. Conclusions

Sensitive techniques are now available for the detection and quantitation of carcinogen-DNA adducts. We have developed specific monoclonal antibodies which recognize DNAs modified by several carcinogens including benzo(a)pyrene and 8-methoxypsoralen. These antibodies have been used in sensitive competitive enzyme linked immunoassays (ELISA) to quantitate adducts levels in DNA from humans with environmental or occupation exposure to carcinogens. In addition, these antibodies have been used in

immunohistochemical studies to localize adduct formation in various tissues. We have also used another approach for DNA adduct detection which post-labels the modified DNA with ^{32}P followed by separation by thin layer chromatography. Adducts are currently being quantitated in lung, placenta and peripheral white blood cells.

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6

Use of Cytogenetics in Detection of Human Exposure to Mutagens and Carcinogens in the Workplace

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1 Introduction

The hazards associated with exposure to carcinogenic and mutagenic chemicals in the environment have become a growing source of public concern. Some two decades of active research have led to identification of hundreds of carcinogens and mutagens in the human environment as well as sources of such exposures. Early detection of hazardous exposures may lead to significant reductions in the risks of adverse health manifestation through proper reductions of such genotoxic exposures.

The identification level primarily includes experimental use of *in vitro* and *in vivo* methodologies. For detection of human exposures at biologically significant levels, considering the complexity of human exposure patterns with interacting life-style, etc., factors and modifying responses, use of cytogenetic methods as indicator of exposure has become feasible. In this respect, occupational exposures are the most reasonable target for studies.

Industrial effluents and products may cause illness also in the general population, but the workers dealing with these compounds are the persons likely to be the first and most severely affected victims of hazardous exposures.

In the following the application of various cytogenetic methods in occupational settings will be discussed.

2. Testing for Genotoxicity of Single Chemicals and Complex Mixtures

During the last years application of short-term tests for mutagenicity in prediction of carcinogenic hazard of chemicals has been widely accepted. Until now at least 13,000 different chemicals have been tested in various mutagenicity assays, mainly in prokaryotic assays and the number is probably at least doubled when unpublished data on industrial chemicals in products safety development are considered. The number of chemicals tested for inducability of chromosomal damage is a much lower, but regulatory guidelines in various parts of the world consider assessment of clastogenicity of the tested compound as a requirement [2].

The use of mutagenicity tests in evaluation of environmental samples has a special significance. The complexity of environmental mixtures, e.g. ambient air samples, very seldom allows the detection of specific single compounds responsible for the effect. Furthermore, the obvious synergistic comutagenic, as well as inhibitory interactions hinder the value of testing a single compound from a mixture. However, the relative inexpensiveness and speed of the short-term tests permits large numbers of samples and their fractions to be tested. Most of the presently available data on complex environmental samples: e.g. polluted air or water, derive from bacterial mutagenicity tests. Future activities should be directed to verification of the results in eukaryotic organisms, among them use of cytogenetic endpoints.

A successful approach to study complex environmental samples includes combination of chemical fractionation and the bioassay techniques, in order to be able to determine the responsible genotoxic component, its primary source or origin, possible spontaneous or artificial transformations and ways to design control technology.

Complex mixtures (of industrial origin and those that occur as a result of pyrolysis or combustion) may be divided into three general classes:

(1) "Commercial mixtures", such as mineral oils, carbon blacks and creosotes, the composition of which varies from sample to sample, depending on the source, method of preparation, etc., but which nevertheless will meet certain physical and (sometimes) chemical specifications.
(2) "Experimentally-generated mixtures", which might include such mixtures as cigarette-tar condensate and diesel exhaust, generated and collected under laboratory conditions.
(3) "Environmental samples", which are, e.g. water samples or airborne materials collected from, for example, the workroom air or as stationary or personal breathing-zone samples.

On-site methods for monitoring ambient air in complex exposure situations at worksites are unfortunately still in their infancy.

Off-site methods are being used increasingly to study potential contamination of occupational environments with mutagenic agents. Because of their relative simplicity, the bacterial mutagenicity tests have been the method used most frequently, but combination with *in vitro* cytogenetics has become increasingly frequent (e.g. Husgafvel-Pursiainen *et al.* [11])

It is essential that proper sampling procedures be used when taking such complex ambient mixtures, in order to guarantee the representativity of the sample with regard to worker exposure. Respirable particles or vapours, although highly critical in human exposure, may easily escape the sampler and cause underestimation of the real hazard [8]. The extraction and fractionation methods may also be overly selective, the mutagenicity may be spread over several fractions or it may be extinguished by the toxicity of the sample [9].

Using the approach of continuous chemical fractionation and short-term assays, the investigator can accumulate information on the actual compounds responsible for the biological effects. Even in view of the difficulties outlined above, therefore, short-term tests provide a possibility for monitoring complex ambient exposure and are thus of importance in following changes in occupational conditions. Short-term tests can also aid in identifying the special hazardous compounds involved and in establishing priorities for more definitive chemical analysis and monitoring, and for further testing in comparative systems, including whole animals, for mutagenesis and carcinogenesis.

A number of precautions should be observed in interpreting experimental data on complex mixtures. Along with the obvious bias that could accompany the choice of nonrepresentative samples, methods of storage and sample treatment may influence the results of any assay. In addition, biological activity in a crude material collected by environmental sampling may be difficult to detect, because of the overall toxicity of the mixture or because of very low concentrations of the active components, or both. Materials are therefore often concentrated and/or fractionated to allow more accurate testing: the possible disadvantages entrained however, include loss or modification of specific components or loss of possible synergistic effects.

3. Cytogenetic Surveillance of Worker Exposures

The main conceptual basis for application of cytogenetic assays, measuring

some type of chormosomal damage in human somatic cells, is the rationale that damage observed in the genetic materials of cells represent high frequency initial events in a process, which eventually may lead to ill-health manifestations. Thus cytogenetic surveillance has the potential to serve as an early indicator, enabling prevention of adverse effects.

Somatic chromosome aberrations have been used for some four decades as an indicator of exposure to chromosome-breaking or clastogenic agents. Most of the early work on induced clastogenicity dealt with people exposed to ionizing radiation [4, 6] and benzene [19]. Since then, over a hundred occupational exposure situations have been studied [3]. However, the number of exposing agents convincingly shown to induce cytogenetic changes in humans is still less than ten [17]. Cytologically recognizable chromosome damage include both structural aberrations, in which a gross change in morphology of the chromosome has occurred, and sister chromatid exchange, which does not result in a change in chromosome morphology, but shows intrachromosomal exchange of material. Promising applications have been obtained with use of micronucleated lymphocytes to account for chromosome damage [10, 12].

Cytogenetic analyses of peripheral lymphocytes have been advocated as a biological indicator of genetic effects on somatic cells (see e.g. [16, 17]). The appearance of induced cytogenetic changes in lymphocytes of exposed persons has already shown itself to be a sensitive indicator of low-level exposures, demonstrated, e.g. by occupational exposures to ethylene oxide [18] or to vinyl chloride [14].

It should be noted that, even though induced chromosome damage in somatic cells is deemed undesirable, the presence of cytogenetic changes cannot be used to predict specific adverse health effects in an individual. However, they do give an estimate of the magnitude of an exposure that could increase the risk of disease in a group of people. Consequently, a cytogenetic study is a relevant method of choice in the evaluation of a hazard in a group of individuals exposed to known or suspected mutagens and carcinogens. Being a laborious task, a cytogenetic study should never be used without some previous knowledge about the clastogenicity of the exposing chemicals.

4. Confounders and Contraindications of Cytogenetic Population Studies

In cytogenetic studies two major types of variations have been documented [1, 17]. The first includes technical factors associated with slide reading discrepancies and culture conditions, specifically with the type of medium, temperature, BudR concentration, which are known to affect the results [13]. Also sampling times can alter chromosome aberration yields, and possibly also SCE incidence through changes in populations of T and B lymphocytes.

The lesions induced in DNA of lymphocytes by chemical exposure that lead to formation of structural chromosome aberrations, sister chromatid exchange and micronuclei must persist *in vivo*, until the blood is withdrawn and then *in vitro* until the cultured lymphocyte begins DNA synthesis. It is

therefore important to score cells after the first division (in the case of chromosome aberrations or micronuclei) or after the second division (sister chromatid exchanges) in order to obtain the best estimate of induced damage.

Scoring constitutes an extremely important element in cytogenetic toxicology. Slides must be randomized and coded to avoid, in so far as possible, scorer bias. Consistent scoring criteria, quality control, and standardized statistical analyses and reporting should be maintained [5, 15].

The second category of variablility is due to conditions associated with the subjects such as age, sex, medication, infections, etc. Individual variations can also be caused by genetic susceptibility to environmental agents (Table 1).

It is critical to obtain a concurrent control group that is matched as closely as possible on innate factors, such as sex and age as well as factors such as smoking status, viral infections and vaccinations, alcohol and drug intake, and exposure to X-rays. Additionally, it is necessary to obtain qualitative (job category, years exposed) and quantitative (e.g., breathing zone air samples for chemical analysis and specific metabolites, if possible) estimates of exposure to the putative genotoxic agent(s) in the workplace. Special consideration should be paid to proper statistical treatment of the results [7].

Table 1. Main confounders of occupational cytogenetic studies and ways to control them.

Confounders	Control efforts
Exposure conditions	
Identification of correct chemical exposure	Factory record checking
Estimate of dose of exposure	Industrial hygiene survey
Individual variations	
Genetic factors	Not known before analysis
Lifestyle factors (smoking, special diets)	Match for smoking, check for drug use, interview in person
Health factors (viral infections, X-ray diagnostics)	Check medical records, interview in person, exclude from study
Culture conditions	
Culture time	CAs: 1st division metaphases only SCEs: 2nd division metaphases only
Culture medium and chemicals	Keep constant, note batches, score M.I. or I/II M
Time between sampling and culture	Keep constant, refrigerate, do not exceed 24 hour
Persistence of the mutagen in the blood sample	*In vitro* experimentation
Analysis and scoring	
Scorer variation	Coded slides, one scorer or quality control slide sets
Interpretation of damage scored	Strict scoring criteria, consultation
Reference groups	
Both methodological and concurrent group controls needed	Individual prematching

Since cytogenetic biomonitoring studies are tedious and difficult in many respects, careful preplanning is very important. Experimental confirmation of the chromosome damaging potential of the exposing agent(s) is a prerequisite to performing human cytogenetic studies.

5. Conclusions

The use of laboratory tests is essential for the assessment of potential human toxicity, so that hazards can be prevented. Laboratory tests are particularly necessary if a new product has been introduced, or if exposure to an old product increases substantially. Situations where the quality of human data is low or difficult to obtain or interpret, also require laboratory test.

At present, the experimental data can and does predict genotoxic risk in man with a reasonable degree of certainty. When supplemented with biological monitoring, results which demonstrate either the extent of exposure or a releation between exposure and early (reversible) effect, the argument for the necessary control of that particular exposure is strong, even in the absence of a definite end-effect detectable by epidemiologic methods. The quantification of the observed risks, however, is problematic.

To date, the risk assessment for genotoxicity have been mainly of a qualitative nature. Somatic effects have been the main interest: information on germinal effects is practically non-existent. In human risk assessment, the weight-of-evidence approach involves the examination of all the available evidence.

The monitoring of cytogenetic effects in humans can be used to provide evidence that environmental agents may have genotoxic potential in humans. If experimental studies have proved that an agent causes chromosomal damage (e.g., SCEs in lymphocytes) and similar responses are found in man, then the possibility of a real genotoxic risk in humans is strengthened.

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7.

Genetic Architecture of Chromosome

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1. Introduction

Knowledge of chromosome structure has been much enriched in recent years due to refinements in biophysical and biochemical tools of analysis. The *chromosome,* as visualized, is a giant complex molecule made up of less complex molecules, the *genes.* The chromosomes of higher organisms are made up of fibrils, approximately 20–30 Å in diameter folded several times to yield the diameter of 100 Å in thickness. It is a continuous DNA protein fibre with alternating condensed and decondensed segments. A single condensed segment of a chromosome of higher organism is often comparable to the entire DNA thread of a microbe. The discovery of highly repeated sequences of DNA, the split nature of the gene, the mobility of the genetic material, the nucleosomal subunits and presence of innumerable replicating units have led to deep understanding of finer details of chromosome structure.

2. Nature of the Thread

The single stranded nature has been confirmed [58, 116, 123], and a single giant molecule runs along the entire length of the chromosome from telomere to telomere associated with histones and other proteins. The packing ratio is approximately 1,000 : 1 in mitotic metaphase. The amount of DNA per chromosome may be very high [58], reaching the value of 10^{10}–10^{11} daltons or even more in a typical mammalian cell. Almost two metres of this DNA are confined within a nucleus, less than 10 μm in diameter. This is one of the best examples of compaction of an immense magnitude [4, 134]. The haploid mammalian genome has approximately 3.0 pg of DNA, containing approximately 50,000 gene loci. In view of the known length of the genes in the mammalian system, with an average of 1,000–3,000 base pairs, the scarcity of the structural gene loci in this uninemic thread [61] can hardly be questioned. In addition, the chromosome structure is quite complex, associated with histones and non-histones, the ratio of which may vary in different phases of growth [153, 154, 172, 173].

Exceptions to this structure are found in differentiating organs, which often show a polytenic constitution. The classical polytene chromosome of *Drosophila* is supposed to be made up of bundles of 2^{10} DNA molecules organized in chromosome arm [7]. Earlier, each band was considered a single gene [8]. Later works proved that more than one gene may be involved in a single band. Evidences indicate that not the puffs, but interband regions are the sites for transcription. Differentiated cells of different lower and higher organisms, such as, endosperm, suspensor, haustoria, antipodals, also contain polytene nuclei arising out of endoreplication [108, 109, 143, 144].

In cereals, the DNA of the polytene antipodal nuclei may have 100-fold amplification [11]. Endoreplication is the means of supply of fresh DNA strands in the differentiated nuclei necessary for continuous transcription, which cannot be achieved by the limited transcribing life of individual strands [154].

3. Replicating Unit

A linear array of potentially independent replicons [22, 158] constitute the

chromosome. It has been specially demonstrated through DNA fibre autoradiography in cultured cells of mammalian tissue [63, 73]. The chain elongation is bidirectional and along the DNA fibre, the initiation of replication occurs at multiple internal sites. Taylor [185] has proposed a model presuming that the size of functional replicons was 100–300 kilobases. A subset of the dispersed repeats may be responsible for suborigins in replicon at early development, as well as during differentiation [183]. Many of the segments may serve as sites for cessation of replication and initiation of transcription. A replicon may represent a cluster of genes, such as, the haemoglobin, including some flanking regions. The master region may trigger the S-phase for cluster replication, whereas the subregions may be more involved in control of genes within the cluster.

4. Nucleosomal Subunits

Ultrastructural and biochemical evidences show that chromosome structure is beaded in appearence [113, 118], each bead being termed as nucleosome. Five histone fractions enter into the composition of nucleosomes, of which four, namely H2A, H2B, H3 and H4 form an octamer, H1 being associated as a linker. The DNA molecule surrounds that octamer with almost 200 base pairs, of which almost 140 base pairs which are highly conserved, are coiled round the octamer. The rest are variable and remain associated with the linker H1 molecule. The eviling of DNA in this structure is non random [86, 87].

Leaving aside about 140 base pairs which go into the core, the 40–70 base pairs of the repeat remain in primary association with lysine-rich histones. From the protein point of view, nucleosome represents the repeats. The relative amount of non-histone chromatin which plays a part in organization of chromosome fibre is more variable than the amount of histone [70, 172]. The interbead DNA in chromatin preparations [118] consists of two pieces of 30 base pairs each, one protected by histone H1 and the other remaining free of proteins or interacting with non-histones [158].

The number of superhelical turns per nucleosome, which is a flat disc, is approximately one/three-fourth, with a pitch of supercoil of about 30 Å and a diameter of 80 Å unit. The size of DNA in the nucleosome corresponds with the *Okazaki fragments* of eukaryotes [187].

Two principal types of interactions characterize the nucleosome. The ionic salt bonds between the basic histones and the DNA phosphate groups are present and the clusters of basic amino-acids are located at the terminal ends of all the nucleosomal [49, 193] histones. The other interaction is non-ionic between histone molecules, and is responsible for maintaining the histone core of the nucleosome [33].

The core of the nucleosome is binary [187] in nature consisting of two flat heterotypic histone tetramers wound by a DNA coil. The heterogeneity of the nucleosome is achieved by the variations in histone fraction. In fact, the histone tetramers perform dual functions. They satisfy the structural requirements for chromosome packing and the variants may play a very important role in the information transfer necessary for gene control. The

DNA in the core nucleosome as noted in mouse liver nuclei [194] shows clear satellite sequences at multiple sites.

Despite increase in repeat length often associated with the onset of transcription or replication as in *Pisum sativum* [133, 188], the nucleosome structure shows remarkable evolutionary stability. This is exemplified in identical ratios of 140 base pairs of DNA and four nucleosomal histones in yeasts and man. Even the conservation of amino-acid sequences in the histones is remarkable. The stability of core histones in evolution, along with its highly conserved nature of DNA with structural repetition, can be correlated with the apparent inactivity of a vast amount of DNA in the eukaryote.

5. Gene Structure

The demonstration of split nature of the gene [34] is a significant deviation from the previous concept. The single gene for a polypeptide is split and consists of coding informational and non-informational sequences, termed as *exons* and *introns* respectively, lying adjacent to each other. The presence of intervening sequences is specially evidenced by the fact that the primary transcript is much longer than the translatable transcript, as noted in globins of man and mouse, immunoglobin and several other genes. The post transcriptional events include cutting off introns and joining of exons by restriction enzymes and ligases. This splicing property is one of the characteristics of the eukaryotic system [47, 167, 168].

The isolation of intact DNA sequences of a gene [141], cloning of complementary DNA and preparation of probes [117], restriction analysis [10, 12], ultrastructural analysis [32] and sequencing [102] have further confirmed the split nature. The introns consisting of highly repeated sequences may occur in multiple copies at intercalary, terminal or initiation points [31, 107], as noted in different organisms ranging from yeast to mammals. In *Drosophila*, the repeats are very long and homogeneous [6] but in yeast phenyl-alanine, gene repeats are rather short [85, 114]. The dispersed introns with repeated sequences may or may not be related [191].

Plant and animal chromosomes do not differ to a great extent [110] in relation to structure of exons, 140 base pairs (BP) sequences being the most common. A correlation has been noted between the size of exon with 50,140 and 200 base pairs and the length of the DNA associated with the linker, core particle and the entire nucleosome respectively. The introns are often suggested as insertion sequences as well. The size of the introns may vary widely from 0.01 to 4.9 kb, though smaller introns, with 100–200 base pairs, are quite frequent. It is however controlled by the information content of the exons. It is claimed that recombination within introns may help in the rearrangement of exons, as in hen lysozymes [4, 23]. It is likely that their differential processing may lead to the reshuffling of exons. The precise significance of introns is yet to be understood.

The primitive nature of introns is indicated in different groups of plants and animals. In maize and soybean, as well as in several other organisms these are more or less located at the same site [146] as the introns of animals. The

length and position of the intervening sequences in beta chain of the haemoglobins of mouse and rabbit between the two segments of DNA represented in the messenger are almost the same [78, 186]. The major introns are 550–600 nucleotides long and the location is adjacent to the sequences coding for 104 amino-acids in mouse and between 100 and 120 in rabbit. It is likely therefore, that the split gene appeared at least 5×10^7 to 5×10^8 years ago and since then the DNA has remained stable. All these evidences suggest that introns are not recent insertion sequences, but they must have evolved at very early stage of evolution [168].

6. Transposons

The mobile genetic elements are specific DNA sequences having the property of movement from one location to another in the chromosome. Such nomadic sequences [9] having a high degree of adjacent repeats, were initially located in maize by McClintock [103]. These have been found later in several organisms including yeast, *Drosophila* and even in the human system [17]. There are two distinct categories [52] of such sequences, namely *insertion sequences* which are short, of about 1,000 bases, and the longer *transposons* which may even be several thousand bases long. The transposition may involve initially replication of the element [112, 147] followed by dissociation of DNA segments and later fusion of DNA molecules [66]. All transposable elements in other location generally have inverted repeats at the ends and they also encode the proteins—transposases necessary for their own transposition [101].

In yeast—*Saccharomyces cerevisiae*, 35 dispersed copies of a repeat family, such as Ty1 gene, have been recorded, constituting nearly 2% of the genome. In culture, the locations of these sequences undergo alteration [19]. The movement of this element in yeast is spontaneous without any activation by mutagens. It is mostly a repetition of 258 base pairs with 100 copies of the gene. In *Drosophila melanogaster*, several types of such transposable sequences have been recorded, the most important of which are 5 kb long copia with direct repeats of 300 base pairs, and 412 sequences—7.5 kb long with direct repeats of 500 base pairs [128, 179] in addition to other sequences like 297, 225 and others.

Four classes of transposable sequences have been recorded in *Drosophila melanogaster* [39]. These are (i) copia type with long direct terminal repeats [136], (ii) FB family of long inverted repeats made up of shorter sub-repeats [127] and (iii) *F* elements with very little free movement [39], though occasional. The fourth type the *P* elements with 31 base pairs of inverted repeats at the ends have a high rate of transposition [136]. This type has the potential of inducing target site duplication [115, 178] in gene transfer.

Various interspersed repeats, such as *Alu* sequences, *Kpn* family, *O* family in *Rodents* and *Primates* show wide genomic dispersion and mobility [68] and are flanked by direct repeats. The *Alu* family is, however, relatively unusual in the sense that even after insertion at a new chromosomal site, the intragenic promoter is retained and is capable of transcription.

The action of transposable elements [112] is controlled by specific pro-

teins such as *transposase*, having the capacity to recognize the ends of the elements. When these proteins are provided, the ends are activated and different target sites are invaded leading to transposition and other DNA arrangements. They evidently bind to the ends of the elements, as also to the other proteins, and as such the cleavage of DNA chains at the point of junction of element and the host DNA is avoided.

The mobile genetic element has a significant bearing in evolution of biological systems [16]. The mobility, pleiotropic effects on surrounding genes, presence of hot spot for integration and capacity to transform other sites into cohesive hot sites may suggest an advanced state of evolution. It may also account, to certain extent, for spontaneous mutation in nature.

7. Repeated Units

The demonstration of multiple copies of identical DNA sequences in chromosomes has significantly influenced the ideas on genetic components of chromosomes [31] and genome change [50]. These multiple copies may be highly homogeneous, as in satellite DNA sequences in *Xenopus*, or may be moderate or minor. Such repeats may be inverted in nature, as in palindromes [22, 23]. The highly homogeneous repeats from algae to higher organisms in eukaryotes may be located in one locus, whereas minor or moderate repeats may be interspersed throughout [83, 156, 160, 164, 176]. The presence of the repeated sequences is responsible for the huge amount of DNA, in the different organisms. In eukaryota, in general only a small fraction of the DNA represents structural genes [108], the rest being amplification of non-coding sequences [53, 54]. The nuclear DNA content varies amongst the organisms over a range of 1×10^{-5} without any increase in the structural genes. In the human system, almost 40% of DNA is repetitive in nature. In several plant species [131, 132, 166, 171], 72–75% of DNA are repetitive, whereas in *Drosophila* only 50% constitute the sequences. Such high degree of repetitive sequences contribute to *C-value paradox*, as manifested in 20–100 pgs of DNA or more in lilies and amphibians, as compared to approximately 7 pgs in man [111, 175], with its highly complex system. Repetitive sequences are present in cell organelles such as chloroplastids as well [14].

7.1. TOPOGRAPHY

Highly homogeneous repeats have been located in different organisms. In addition to *Xenopus*, ribosomal RNA sequences contain long homogeneous repeats. Such homogeneity has been noted on human acrocentrics [59], *Y* chromosomes [29] in man and in *Drosophila*. In *Xenopus*, the repeats are often flanked within the two large albumin genes, in addition to being scattered within and around the four vitellogenin genes. In the plant system [41, 124], satellite DNA is present in knob-heterochromatin of *Zea mays* as well as in *Trypsacum dactyloides* [41]. Telomeric heterochromatin too is often rich in repeated sequences [2, 95, 96]. Highly repeated sequences are present in the nucleolar organizing regions involving 80S and 20S rDNA of the different organisms, as well as in 5S sequences [51, 72, 126].

Accessory or supernumerary chromosomes, as in *Scilla autumnalis*,

Secale cereale and *Secale montana* [80, 106, 120, 170] are the cytological embodiment of the heterochromatin and rich in highly repeated sequences. Both moderate and minor repeats, more or less, remain interspersed in between the unique sequences [38]. Nearly 40–50% of the genome of millets consists of repeats [60, 176]. The intercalation of small and moderate repeats is more or less universal in all organisms and their variability too, such as in wheat, has been recorded [2].

In addition to such interspersion, the presence of repeated copies in intervening sequences or introns [6, 114], transposons, universally in nucleosomes [179] and in flanking regions of replicons [185] and spacers [2] has already been described [169]. A non-transcribed spacer group of mouse ribosomal genes-200 base pair long and located between alpha and beta globin gene, has been utilized lately for screening of human gene library [189]. The conserved nature of the repeated sequences in spacers has been found in their similarity in *Xenopus*, pigeon, slime mould and yeast.

7.2. EVOLUTION

The mechanisms of evolution so far visualized includes saltatary replication [25], unequal crossing over [139, 177] and transposition including insertion [18, 43, 162, 163]. In Syrian hamster, shorter repeats are supposed to have evolved from fractionation of the longer repeats [105]. The accumulation of short repeats is a very common event in the evolutionary cycle and it may even represent duplication of regulator sequences. Several repeated sequences, both coding and non-coding ones, often show intraspecific homogeneity, indicating concerted evolution. Characteristic sequence variability has been recorded in histone, rDNA, globin, immunoglobin genes [5, 15, 24, 195], as well as in SAT DNA families of *Drosophila* and mouse [195]. The conservation of the repeated sequences in primates, including man, gorilla and chimpanzee, has been well established [29, 35, 40, 137, 148, 150]. A progressive homogenization of repeated sequence families during evolution, through continued turnover of sequences, has been suggested. Such a process can be mediated by gene conversion [15], transposition [18] and unequal exchange [177]. It is quite likely that different mechanisms have been involved in the origin and evolution of repeated sequences.

7.3. IMPORTANCE

The significance of such sequences is still not clear. Their conservation in diverse groups of organisms even in intragenic introns, as in *Tetrahymena* [82] may indicate a selective advantage. Several non-specific functions involving cell and nuclear size, cell and nuclear volume, chromosome cycle, generation time, duration of meiosis and the padding to keep the chromatin in folding state have often been attributed to repeated sequences, otherwise termed as *nucleotypic* DNA [3, 10, 11, 53]. The highly homogeneous repeats—the *satellite DNA* has often been associated with various organisms including *Xenopus*, pea and *Drosophila* [50, 51, 121, 120]. In *Drosophila* it has been found to be associated with membrane, suggesting transport func-

tion. The role of interspersed moderate repeats in repair has been demonstrated in *Lilium* [37, 65] and in the conservation of *Pachytene DNA* [92, 97, 98]. Their importance in the regulation of chromosome structure in folding during pairing too has been recorded [79]. In replication, in *Xenopus*, a subset of the dispersed repeats may act as initiation points in early development and later during differentiation [183, 185]. In haemoglobin clusters, where flanking repeats are present, they may be involved in coding of methylalanine pattern [184]. In the *Y* chromosome of *Drosophila*, there is a remarkable correlation with the position of male fertility factors and chromosomal blocks, rich in repeated sequences [41]. In the sea urchin histones, in all the five genes repeated sequences have differential capacity of transcription [67]. In maize, the repeated sequences are associated with specific classes of heterochromatin, having particular meiotic effects [41]. In mammalian genome, the continued interspersion of repeated and single copy [68, 138] sequences and their mobility may suggest important genetic functions.

The repeated sequences may represent reservoir of new sequences [138] and loci for accumulation for mutation [154, 162]. The interspersion may allow flexibility of units. The presence of multiple copies may meet the requirements for rapid histone synthesis during embryogenesis [36]. The microtubules of hog brain non-histone protein bind very effectively to mouse satellite DNA *in vitro*, suggesting some specialized functions [192].

Intergenic conversion and reappearance of suppression alleles are facilitated by exchanges in repaets in fission yeast [48]. Recombination occurs within large repeated gene clusters in globin region of caucasian human systems [100]. In *S. purpuratus* [99] RNA hybridization is preferential if non-repeats are associated with repeats.

The palindromic repeats have diverse functions. They replicate under specific conditions and protein synthesis is affected at different levels through such sequences. Functions attributed to them include recognition system both at DNA and RNA levels involving deletion and translocation [84], cleavage sites, termination of transcription and binding of regulatory proteins, and attachment of chromosome with each other helping in information transfer [22, see 95, 96].

The repeated sequences have been extensively observed in spacer regions in several organisms [2, 26, 88]. *In vivo* experiments indicate their role in initiation and termination of transcription as well as their association with heavy RNA synthesis. Large amounts of repeated DNA have been recorded in accessory chromosomes [41], which are cytological embodiments of repeats. The adaptability of accessories in certain alpine species, like [106], *Arisaema* [152, 159] as well as experimental demonstration of the adaptibility in *Allium stracheyii* are also on record [170].

7.4 POSSIBILITY OF MANIPULATION

The repetitive DNAs in chromosomes are additional gene sequences, which due to their property of amplification, dispersion and mobility confer flexibility to the species [162]. As they do not necessarily involve structural

genes and are present in multiple copies, the manipulation at such sites may not lead to deleterious effect on the organism. Their function as spacers or insertion elements may suggest that these loci may be ideal sites for restriction enzyme operation. Different restriction sites too have been located within a single repeated segment.

To some extent, the accessories [171] are comparable to self-replicating plasmids of prokaryotes, in spite of their location inside the nucleus. There are ample records of B-chromosomes multiplying outside the nucleus forming micronuclei [106]. As in case of plasmids, with judicious manipulation in suitable materials, B-chromosomes may serve as vectors for transferring genes from one organism to another. Such a possibility is borne out by the fact that they may sometimes carry euchromatic or more precisely, structural genes. If such a vector system is established, the transfer of genes from one organism to another through such accessories may not only be a theoretical possibility [165–167].

7.5 FUNCTION

Several epithets have often been applied to reiterated sequences. They have been termed as *Selfish DNA* [119] in view of the capcity of duplication in a congenial cellular environment without any evolutionary function. Such selfish DNA having the capacity for mobility between different chromosomes has been termed as *parasitic* and when beneficial, as *symbiotic* DNA [44]. These are incidental to high pressure mutation, for which the term *incidental* DNA is applied. Some of the genes, on losing their functions acquire the term *pseudogenes*, a term which is not appopriate as they become functional following further mutation [130].

In view of the demonstration of several non-specific and regulatory functions as well as their conservation in evolution, it is not justifiable to consider such sequences as *selfish* or *junk* or *trivial*. The highly amplified sequences, whether they are present in introns, transposons, spacers or in any of the intercalary regions, have the common property of *amplification*, *dispersion* and *mobility*, the last property being demonstrated in some and undemonstrated in others. In view of all these properties, the term *dynamic DNA* [162, 166] was proposed, implying their dynamic influence on vital functions of the cell. These sequences do not normally code for structural proteins but influence their structuring and functioning. The non-specific functions which they perform are subject to physiological influences, which are controlled by their flexible properties involving amplification, dispersion and mobility. The dispersion is possibly a strategy against complete elimination.

8. Molecular Manifestation of Chromosomes at the Cellular Level

In the study of chromosome structure, methods have been devised to study the sequence complexity of DNA and the functional segments at the cellular level. The chromosome banding is based on the principle that the single strand of RNA and DNA can recognize and pair with the complementary base sequences. In a denatured duplex, highly repeated sequences undergo rapid reannealing. The reassociation kinetics provide an index of sequence com-

plexity. This protocol for molecular hybridization, i.e. denaturation and reannealing, if followed by staining with different dyes, specially giemsa, gives intense positive reactions at similar segments, otherwise showing repetitive DNA. Such bands obtained following denaturation, renaturation and giemsa staining have been termed as *C*bands [3, 71]. The banding techniques thus bridge the gap between molecular methodology and cellular techniques [155, 174]. Caspersson and his colleagues recorded differential fluorescence of chromosome segments with various fluorochromes under ultraviolet light and obtained distinct banding patterns, known as the Q band [20, 21, 142, 149]. Saline, trypsin and various other chemical treatments followed by Giemsa staining cause *G* banding [90, 91, 93, 94, 142], not necessarily requiring de- and re-naturation [64]. *Reverse* banding (R) by Dutrillaux [46] requires controlled heating. *O* banding based on orcein proceded by trypsin or acid or SSC-treatment, yield bands in plant systems [23A, 89, 155]. Acid extraction method is employed for nucleolar organizing region or *N* banding [56]. The available evidences indicate that the unique sequences carrying genetic informations are mostly located in R bands. In the human system, this technique has been utilized for locating linkage studies [135] as in 3P, 6P, 11Q, 17Q and 19 chromosomes of the human complement [148, 150].

Several causes have been attributed to the occurrence of bands including repetitive DNA, difference in the base composition of DNA, degree of packing of DNA or the nucleoprotein component [27, 140, 174, 180, 181]. With *Q* banding, the fluorescent aminoacridine nucleus becomes intercalated within the double helix and ionic bonds are formed between basic nitrogen atom and DNA phosphates [20, 21]. The alkylating side group binds covalently with guanine [104], leaving aside *Q* banding in both *Q* and *O* banding. The DNA protein linkage possibly plays a very significant role [27, 28]. Along with histones, the importance of non-histone-protein in the manifestation of bands cannot be overrated.

The identification of functional segments of chromosomes correlated with their molecular composition is achieved through *in situ* hybridization [37]. It involves denaturation of DNA, preparation of a labelled complementary DNA or complementary RNA through specific enzymes and hybridization of complementary DNA or RNA sequences on the chromosome smear, which forms either DNA–DNA or DNA–RNA duplex. It is possible to identify the moderate or highly repeated sequences and the functional loci, such as ribosomal RNA. The technique has further been modified, to map a single copy sequence in some of the mitotic chromosomes [65] as well as to analyse the ultrastructure of specific active genes [62, 75, 76]. Probes have been developed differentiating discrete oocyte clusters from somatic clusters [62, 126], occurring at or near the tips of the chromosomes. Following this method, in wheat, a highly repetitive sequence has been located at the interstitial region or telomeres [74]. Banding technique associated with hybridization has also been successfully employed for the identification of segments in different organisms including both plant and human chromosomes [8, 42, 51]. In hybrids of wheat, rRNA gene sequences have been located at the nucleolar organizing region [76]. Ribosomal DNA gene probes have also

been utilized for *in situ* hybridization with polytene chromosomes and suspensor of *Phaseolus* [45].

9. Future Scope

The advances in knowledge owe undoubtedly to refinements in methodology. But none of these techniques is free from limitations [126, 174, 181]. Cell fusion is a powerful tool in mapping gene loci in chromosomes, but depends greatly on a chance factor. Banding techniques and the patterns they reveal are useful, but their mechanism is not fully understood. The extent to which the reaction involves AT or GC component of nucleic acid or the protein is to be finally resolved.

The significance of the repetitive sequences has not yet been adequately assessed [162, 165]. The unravelling of their functions would lead to a better understanding of functional sequences in chromosomes. The *in situ* hybridization technique is undoubtedly a powerful tool, but it can identify mostly repeats, with certain exceptions of uniques. Further refinements are needed to delineate the minor repeats or even unique sequences, which may ultimately lead to the correlation of structure and function of each chromosome segment.

The complexity of chromosomes structure, with the arrangement of sequences in multiple copies in split genes, in nucleosomes, and in a linear array of potentially independent replicons, has been resolved to a great extent. With the gradual emergence of complexity in evolution, evidences have been gathered to show a variability in chemical make up, mainly involving DNA amplification and protein content during organogenesis with basic genetic skeleton intact [167]. Such amplification has been noted in sea urchin embryo, root of *Vicia faba*, and a number of other species in their differentiated organs [122, 125]. Variability in chromosomal protein component has been noted during embryogenesis and organogenesis of various plants and animals [13, 77, 129, 137, 145], including the chromatin associated protein in human sperm.

Along with the origin of the complexity of the chromosome structure and the concomitant evolution of the mechanism of its operation, there was the development of system of dynamic changes in structural pattern during organogenesis, maintaining the basic genetic structure. This remarkable genetic property of the chromosomes enabling them to change their structural pattern during development is one of the crucial factors for control over all aspects of differentiation. The evolution of both complexity and flexibility has been the principal events controlling sequential growth and development in high eukaryota [151]. The mechanism through which this inbuilt dynamic pattern is programmed and genetic control is maintained, is yet an unsolved problem. The expression of an apparantly single character with various constitution of tissues and organelles is triggered and subsequently operated from different gene loci. An understanding of the mechanism through which this triggering of different loci distantly located is synchronized, is still not understood. Chromosome manipulation *in vitro* involving

different loci on different chromosomes may ultimately lead to the solution of this intricate problem.

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8

Some Botanical Consequences of Tectonics and Orogeny

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1. The Gramineae of South Asia

A study of the members of the major plant family, the Gramineae/Poaceae, provides an opportunity, in South Asia in particular, to relate or calibrate the various botanical processes involved in their evolution with regionally significant geological events of a tectonic or orogenic nature. This chapter is primarily concerned with the known or presumed facts of the evolution of cells and nuclei, which must be regarded as fundamental to the morphological and physiological manifestations of botanical evolution, especially speciation, over the geological ages concerned. The emphasis is upon what cytology can contribute to such a broad evolutionary study, rather than upon the minutiae of cell and particularly nuclear structure and behaviour, which are primarily the interest of laboratory cytologists—using appropriate species of the Gramineae as botanical *Drosophilas*.

The grasses of South Asia have already been classified into the subfamilies, tribes, genera, species and other groupings accepted by the taxonomists [12, 37]. It is, however, possible to propose an alternative or supplementary classification, distinguishing in this between species that arrived in South Asia from other regions on tectonic plates or along migration routes, or which evolved (speciated) on the plates or later within the region, in the infinite variety of palaeoclimatic conditions which were created by the geological events, which occurred in or adjacent to the South Asian region. A major fact to be considered in this connection is that, the origin and early evolution of the grass family had not occurred when the rafting of the South Asian Plate from East Africa had begun.

The groups within the gramineous flora of South Asia may be briefly summarized as follows:

a) species of East African/Madagascan affinity, which crossed on the South Asian Plate between the beginning of rafting (late Cretaceous) and collision with Laurasia (Eocene) (See Sec. 2.1 below);

b) the species of the Deccan Plateau which evolved from the above in the rainshadow of the eastern crest of the Western Ghats,

c) xerophytic species of the north-west of the region, which arrived from north-east Africa and the Near East after the establishment of new drought conditions in or following the Eocene;

d) groups of species which became established down to specific contours in the Himalaya and associated ranges, having come from the north (Siberia through Innermost Asia) or the west (Irano-Turanian region).

The grass evolutionist asks the cytologist for evidence which may be available or is still to be found, of genotypical response (especially cells and nuclei) to the great environmental changes which occurred from the geological period under review. What are the cytological criteria for primitiveness, to supplement the morphological and physiological; what are the accepted original stages of evolution of polyploidy [83] and their changes of chromosome complements, and of apomixis; what is the evolutionary significance of isozymes in the cells of wild and cultivated grasses; how do plants (cells) change their photosynthetic pathways as a reaction to progressive exposure to

changing environments and altitudes, particularly to ever-increasing aridity in hot and cold climates; what is the cytology of endemism? All these processes of cell structure, physiology and reaction are concerned in the ultimate genetical nature (adaptation) of progeny which arose from the millennia of speciation, which occurred in the grasses of South Asia.

After consideration of some of these questions, necessarily brief in the space available, * case studies relating to some of the above groups of grasses or to individual genera and species, wild and cultivated, will be presented to indicate trends in current research on the cells and their genetical expression and response.

2. Origin, Evolution and Speciation

2.1 GEOLOGICAL AGE AND PLANT SPECIATION

This chapter is based on the acceptance of the theories of plate tectonics rather than those of the expanding earth [58], which are most difficult to reconcile with the known and theoretical aspects of plant biology. The geological facts relating to the movement of the South Indian Plate and the sequence of palaeoclimates are known. From the late Cretaceous onwards, the plate began its long rafted journey (over 50 million years) from a position adjacent to Madagascar and East Africa up to collision, following a 25° change of main axis, and the subsequent probable reduction in overall length of some 2,000 km [174], with the landmass of Laurasia in the Eocene.

The choice of the Gramineae for this study is appropriate, because the whole evolution of that plant family took place during the same period. It is stated that the Gramineae first appeared in the Paleocene, followed in the Oligocene by fossil spikelets of *Stipa*, coincident with the first appearance of high crown teeth in fossil mammals [34]. In calibrating the geological history (and consequent palaeoclimatic history) with the origin and evolution of one plant family, it is necessary to consider current theories regarding the possible ancestors of the Gramineae and the development of primitive forms. There is a need for data on the cytology and consequent genetical behaviour of the first primitive and later more evolved forms of the Gramineae. For example, what are the morphological, physiological and cytological criteria for primitiveness in monocotyledons and/or Gramineae in particular (see Sec. 2.6)?

At this point, it is necessary to refer in passing to alternative views of the place of origin of the grasses—that of C.E. Hubbard, N.L. Bor and repeated by Clayton [34] that the grasses arose in or near the edge of the tropical rainforest, and that of G.L. Stebbins [144] and personal communication (1986), that the grasses arose in the semi-arid savanna and migrated back to the rainforest or

*The author had planned to take up his long-term study of the Gramineae of Asia in 1987, beginning with a contribution entitled: A Geological Classification of South Asian Grasses, to the second Conference on the Palaeoenvironment of East Asia, Centre of Asian Studies, University of Hong Kong, 9–14 January. He then envisaged starting a book-length work, which would include the conclusions he had reached on the origin, evolution and present status of the Gramineae of Asia, based on over a lifetime's study of the subject, and which would also discuss the views of colleagues from all world, in the form of personal communications, reprints and publications.

out into other semi-arid and more arid environments. Most authorities relate the origin of the grasses to the Commelinales in general and the Flagellariaceae in particular, plants of the tropical forests [33].

"Now the Bambusoideae, a subfamily defined by certain anatomical peculiarities such as fusoid and arm cells, is both tropical and primitive. Primitive in the sense that some genera have incomplete suppression of axillary buds in the inflorescence and spikelets, and the flowers may retain trimerous symmetry. Among them is a group of small tribes (e.g. Olyreae) known as the bamboo allies, which paradoxically combine the primitive and baroque. This collection of curios, apparently the relics of ancient departures from the mainstream of grass evolution, gives some hint of the diversity that must once have existed in the ancestral stock. They are mostly insignificant broad-leaved inhabitants of the rainforest ground layer, which are often mistaken for other forest families such as Zingiberaceae. By contrast, their near relatives, the true bamboos have become successful competitors in this environment by developing woody tissue and adopting the form of trees."

The most likely grasses which first moved from the forest into savanna, "belong to the subfamily Arundinoideae, for this is also primitive; primitive in the sense that it lacks distinctive features, but seems rather to represent the lowest common denominator of subsequent subfamilies. Its notoriously difficult taxonomy suggests that it is now reduced to dismembered fragments around a core which has become extinct.

"Subfamily Chloridoideae is easy to place, for it abuts onto Arundinoideae, and indeed the boundary between the two is difficult to establish. It ushers in a new development, the Kranz syndrome. This is a set of anatomical characters associated with C_4 metabolism, in effect an extra loop in the photosynthetic cycle (the basic form of which is known as C_3), that renders it more efficient in high temperatures [154, 160]

"Subfamily Panicoideae presents more problems. It has also evolved the Kranz syndrome, in fact two different versions of it (the MS and PS types of Brown, [15]), but some genera of Paniceae still retain the older non-Kranz anatomy and C_3 metabolism. Moreover, there are no direct links between the main tribes, Paniceae and Andropogoneae. The situation is still rather confused.

"Finally, subfamily Pooideae seems to represent a new venture for the grasses, an adaptation to cold climates and invasion of the temperate steppes. It has retained the primitive C_3 pathway, for the more advanced form offers no advantage in a cool climate. This suggests a relationship with Arundinoideae, a few of whose genera have shown their ability to penetrate deeply into the temperate zone" [33].

In the present context, one must ask whether the initial stages of evolution of the Gramineae had begun before the South Asian Plate had been biologically separated (that is, 2,500 km away) from the African mainland and Madagascar, and if so, how far had it progressed from the primitive begin-

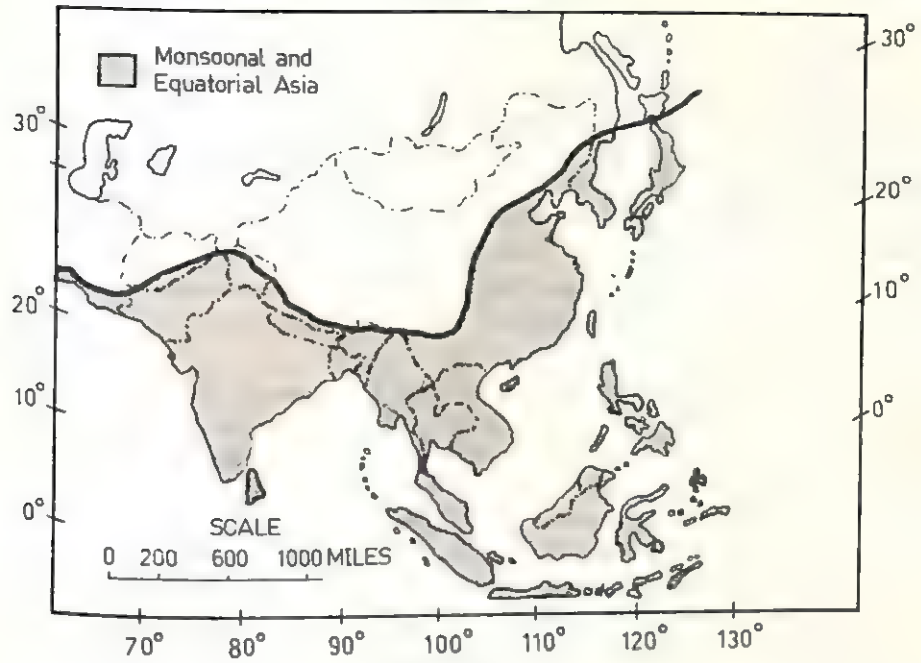

Figure 1.

nings. Did subsequent evolution through the early stages of the Tertiary follow parallel lines on both the African mainland [13, 20, 21, 52] and the Plate?

Were the several cytological and genetical phenomena of the South Asian grass flora introduced at progressive stages with exposure to the ever-increasing moisture stress characteristic of the geologically induced palaeoclimates—abnormal meiosis, aberrant chromosome composition, polyploidy, apomixis, adoption of new photosynthetic pathways, types of isozymes, etc? In brief, how much of the speciation which occurred is a reflection of genotypic response to continuously changing environments, and how much to adjustments (progressive or mutational) in the basic cytological composition and genetical behaviour of the species involved [75, 149]? Nagendra Prasad and Janaki Ammal (1985) found that speciation "is occurring at a fantastic speed in the diploids of Silent Valley, Kerala", a relict of original virgin forest. It would be interesting to conduct observations on cell genetics among the Gramineae (particularly the possibly ancient aquatic or semi-aquatic types described for Sindhudurg District in the Western Ghats) [1].

In assessing the significance of palaeoclimatic change in the evolution of the Gramineae, it is necessary to consider the possible sequence of palaeoclimates, which existed on the plate before and during its movement from Africa to Laurasia. Were there, then as now, arid regions in Madagascar [115] and therefore also on the Plate? Were vestiges of these initial arid regions retained as the Plate crossed the Equator, or were they and their vegetation cover replaced by one adapted to a climatic sequence subtropical/equatorial/subtropical?

2.2 STRESS

Consideration of the effect of stress on speciation [82] involves a decision between two markedly contrasting outlooks on the whole question of the reaction of the genotype to environmental change.

In their study of floral differentiation in *Triticum* Frankel and Roskams [49] state that "the reproductive organs of plants as a rule are effectively buffered against genetic and environmental perturbation" and that it is this constancy which is the main difficulty in research into the genetic and physiological control of differentiation of reproductive organs. Variations which might help to make the normal better understood are thought to be rare and destructive.

This view is that, abnormalities at meiosis are primarily expressions of the genotypical composition of the plant—that they are due to the action of genes governing degrees of *absence* as well as *presence* of biochemical characters or substances, to so-called innate genetic imbalance, or sterility loci and alleles determining sterility, to autonomous genetic systems of the organelles and interaction between mitochondria and nuclear genes, to a locus (Ph) on the long arm of a chromosome [41], to the genetic regulator [72] to the effect of the 5BS chromosome on the duration of meiosis [7], or to the location of the Ba gene which plays a major role in the control of normal floral development [48].

On the contrary, it is necessary to ask, first whether and under what conditions and in which particular species, abnormalities during and after meiosis can be regarded primarily as an expression of reaction to various forms and intensities of environmental stress. That stress would be caused by insufficiency of water and of soil nutrients and by the presence of high and fluctuating temperature, before and during the period of flowering. Stress operates on the uptake and movement within the plant of the crude nutrients, which are precursors to the complex biochemical substances essential in the formation, development and maturation of the germ cells. The nature, direction and rate of that movement are governed by the specific vascular structure in the stems and within the flower or inflorescence.

With this interpretation, physiological, morphological and biochemical factors are seen as governing the degree to which the genotype or the particular genetic system may be permitted or induced to express itself in overall reproductive efficiency and in the production of normal or abnormal gametes and zygotes, according to the combination of conditions obtaining at any particular time and place.

Unfortunately, it would appear that those who study cytological reactions and genetical consequences of various forms of stress stop short of examining conditions of nuclei and chromosomes in mitotic and meiotic divisions. Petolino and Collins [106] have reviewed the literature (182 references) on cellular approaches to the obtaining of response to environmental stress in plant breeding; stress due to water deficiency (see [70]); excessive temperature and the presence of excessive salt and metals. Reference is made by Petolino and Collins [106] to the use of cellular selection techniques by cell lines of haploid and diploid *Nicotiana* and *Capsicum* with enhanced response to

chilling temperatures. The cellular selection resulted in alteration of mitochondrial activity, known to be related to temperature tolerance in whole plants [40]. It appears that the increased chilling response was epigenetic in nature. Exposure of tomato plants to low temperatures during pollination and fertilization resulted in the differential survival of the gametophytes with cold-tolerance genes. Only those pollen grains able to germinate and elongate under low temperature would function in fertilization and contribute genes to the progeny [175].

Important research on water stress is in progress at the Central Arid Zone Research Institute, Jodhpur, Rajasthan, regarding the effects on both growth and yield, but again, without reference to the normality or abnormality of mitotic and meiotic cell divisions basic to the expression of this measure of economic production [51, 79, 80, 165].

Canadian workers have evaluated tolerance of cobalt, copper, nickel and zinc in clones of *Agrostis gigantea* [68] and in *Deschampsia caespitosa, Hordeum jubatum* and *Poa compressa* [116].

Many observations have been made on plant growth and reproduction without sufficient concern for the basic mitotic and meiotic cell divisions to which these are the ultimate manifestation. Grime [55, 56] has applied his hypothesis of plant strategies to experiments on flowering response to drought stress in species of *Urtica* [11]. Grime has proposed that differences observed in plants coincide with lower xylem water potentialities and higher mortalities in *Urtica urens,* and is consistent with plant strategy theories reflecting life history and reproduction to frequency of habitat disturbance.

Rozijin and van der Werf [120] have studied effects of drought treatments applied in different stages of the life cycle and allocation of biomass in the vegetative and reproductive phases of winter annual species of the genus *Aira.* Biomass allocation showed greater differences between species than between the treatments, especially in the reproductive phase. The proportion of biomass allocation to reproductive organs was affected by the drought treatments, but there is no reference to actual cytological response.

2.3 ISOHYETS AND CONTOURS

Boundaries between major geological ages are of great significance in biological evolution—for example, the Cretaceous/Tertiary mass extinction of fauna, major changes in type of vegetation (gymnosperms to angiosperms). But these are not so relevant to the history of the Gramineae in South Asia, as the many boundaries between one type of ecoclimatic condition to another. These have been and are still being of great significance in the cytogenetical evolution of the grasses. Examples are:

a) isohyets of the north-west of the region, ranging from the low, spasmodic and biologically ineffective rainfall of the western zones credited with, say, 10 cm of rainfall per annum, to the zones with reasonably adequate monsoonal rainfall;

b) the crest of the Western Ghats, where rainfall drops so markedly on the eastern side;

c) altitudinal contours in the mountains of the north and in the Nilgiris and other lesser mountains.

After the initial creation of new boundaries following the catastrophic introduction of arid conditions in the Eocene and the later establishment of the rainshadow on the Deccan Plateau, the borders between arid and semi-arid and the more humid would have fluctuated in relation to relatively minor climatic changes. The high rate of speciation caused by the effect of excessive physiological stress on the genecological composition and behaviour of grasses following the Eocene became much reduced with the establishment of a reasonably stable but fluctuating monsoonal pattern. The efficiency of the light rains of the arid and semi-arid areas became much less later, with the advent of man as an agent in general devegetation, and the renewed exposure of grass species to high intensities of desiccation at the ground level. The effect of the environment on the genotypes at this time was expressed by increase in the proportion of progeny of perennials adapted to greater stress, or by the increase in the proportion of drought-escaping annuals, now cytologically and genetically fixed and unable to revert to the perennial state.

There are in the Himalayas and associated ranges a number of contours which represent climatic and vegetational boundaries (see [86, 110, 111], for a comparison with European conditions), which have had and to some extent still have, a considerable significance in grass distribution and speciation. In the interpretation of species distribution along altitudinal gradients [66], three borders in these mountains are of primary significance and call for extended research on the cytology of the grass species involved:

a) the border between monsoonal and humid-temperate environments

b) the border between humid (cold) temperate and the alpine semi-arid (cold)

c) the upper border between alpine semi-arid and high alpine arid.

The most significant of these boundaries in the present connection is the first, generally around 2,000 m contour. This may be said to be the limit of tolerance of humid temperate conditions by monsoonal species and the lower limit of tolerance of monsoonal conditions by the grasses of the humid temperate environment.

Basing conclusions on the study of the cytology of the Gramineae from lower to higher altitudes in the north-western Himalaya, north of Chandigarh, P.N. Mehra (personal communication, 1973) considers that variability in populations of a species at both the taxonomic and cytological levels is most likely, as one approaches the ecoclimatic borders of its distribution; in other words, that stress at the peripheral portion of distribution may accentuate the intensity of variation. Abnormal meiosis is ascribed to such causes as environmental factors, extensive vegetative multiplication, browsing by animals, and "genomic instability as reflected in the various meiotic aberrations and subsequent production of sterile pollen." It has, however, been noted that certain variations in ambient temperatures do affect meiosis, and lack or excess of critical elements in the soil may well do likewise.

Mehra and Ramanandan [90] recorded meiotic aberrations in different degrees in 13 out of 28 species and recorded comparative morphology of the intraspecific cytological races of 28 species of grasses (Pooideae) of the west-

ern Himalaya, collected at elevations ranging from 1,500 to 4,300 m. These are species of temperate genera, occurring here at one of the southern limits of their south Asian distribution, few of which set seed; if they do so, the seeds are mostly sterile.

A companion paper on the Panicoideae [91] describes the cytology and distributional pattern of 23 species at or near their altitudinal limit on the monsoonal/temperate border in the western Himalaya (Chandigarh/Simla); in only five of the species examined is some degree of disturbance at meiosis recorded, an interesting contrast with the Pooideae. Again, in a study of species of the Andropogoneae and Paniceae in the central and eastern Himalaya (Nainital and Darjeeling respectively) Mehra and Sharma [92, 93] report a high degree of disturbance of meiosis only in a pentaploid of *Pennisetum orientale*. Among the temperate species of the western Himalaya, partial or complete breakdown of meiosis is noted in *Poa alpina*, *P. bulbosa*, *P. nemoralis* and *P. pratensis* [94].

For the further clarification of the nature of the genotypic response to the climates of increasing altitudes in these mountains, it will be necessary to conduct cytological field studies of grasses of temperate adaptation along altitudinal transects. These would extend from the lower contours at the limit of their tolerance of monsoonal conditions, up through elevations of maximum adaptation promoting normal growth and reproduction, to the higher levels where physiological and cytological reactions are limited by intolerance of the semi-arid cold.

2.4 ALTITUDE

If any species of the Gramineae were growing in the southern parts of Laurasia during the Oligocene and subsequent geological ages, along the northern shores of the Tethys, they must have experienced a very unusual sensation, the rise of the ground beneath their feet, as it were. They progressively faced the uplift of their environment from an initial average 1,000–2,000 m altitude in a tropical to subtropical environment, to altitudes extending up to 8,000–9,000 m in a humid-temperate to cold-arid environment.

In plants facing such environmental changes, disruption of meiosis (reproduction) will probably precede cessation of mitosis (growth) in the somatic tissues. To cope with this situation, plants would need to have the capacity *either* to migrate down to elevations which could still provide the environmental conditions to which they are adapted physiologically and genetically, *or* they should have the capacity to produce, within the range of variation in their progeny, a certain proportion of viable offspring, better able to grow and reproduce in progressively more extreme environments, variation within the total genotypical scope of the species.

Escape mechanisms would include characters of types with greater physiological response (speciation), higher polyploidy from lower ploidy populations (see Sec. 2.7); types with changed photosynthetic pathways, or capacity to produce certain porportions of annuals (therefore winter- and/or drought-escaping) from predominantly perennial and thus less viable progeny [113].

2.5 ENDEMISM IN THE GRAMINEAE

In discussing speciation as the main driving force of chorology, Clayton [31] states that "one of the traditional approaches to the problem is through endemism, and attmepts to distinguish between centres of origin and refugia. The frequency of one-country endemics has been mapped by Clayton and Cope [35], revealing classical centres in Fouta-Djallon and Ethiopia, but elsewhere the pattern is very confused. The highest counts occur in South Africa, Madagascar, Peninsular India, South-East Asia and Australia, where isolation is clearly an important factor. Otherwise, they are strongly correlated with mountain topography (the pediplain of the Congo-Zambezi watershed is an unexplained exception), and form long chains rather than discrete centres. In short, the concept of endemic centres, at least in its simple form, is shown to be somewhat naive and not particularly helpful. The geography of speciation remains an open question, but I would suggest that the striking difference between the mountains and the plains lies not so much with active speciation in the former, as with ruthless extinction in the latter."

Endemics are regarded as having one restricted region or area of distribution. There are two types [81]:

a) a young species or genus which may not yet have attained its maximum area as determined by its dispersal barriers—an endemic in the strict sense;

b) old or relic species, occupying a much smaller area than before, an element that is surviving but not contributing to floral evolution—an epibiotic.

Members of the Botanical Survey of India have contributed a number of studies on endemism and patterns of distribution of angiosperms (including Poaceae) in various parts of India [100]. The nature of endemic taxa is assessed on the basis of [101]:

1) taxonomically isolated endemic taxa,
2) disjunction in their distribution, and
3) presence of fossils.

Families with the largest representation of endemics are the Fabaceae and Acanthaceae, followed by Poaceae and Asclepiadaceae [102]. The survival of palaeoendemics in the Eastern Ghats is dependent on biotic, climatic and edaphic factors, as well as on the genetic structure and past history of their populations, in accordance with the gene-pool/niche interaction theory [148]. The isolation of narrow endemics due to various environmental barriers is more pronounced in the Eastern Ghats than the Western Ghats, where there is a more continuous mountain system, and a more humid climate. The endemic plants of Maharashtra have been listed by Almeida and Almeida [2] of India and Sri Lanka by Sarkar [127].

There is no doubt that a comprehensive study of the grass endemics of India, dealing especially with habitat, geological age and cytology, would produce data highly relevant in the present context, as that dealing specifically with endemics of gypsum deserts in North America in particular [161]. Gypsum is a common surface evaporite in warm or hot regions of low rainfal throughout the world. Enormous deposits were formed during the Mesozoic, 150–240 myBP, but these were subsequently covered by the calcareous or

siliceous deposits of the Cenozoic. Tectonic and erosional forces have re-exposed this fossil gypsum or its anhydrite form [4], for example in the Sierra Madre region of Mexico. "Strange and bizarre plant endemics tend to develop upon such substrates, especially where the surrounding flora is rich and the gypsum outcrops of long standing. Genera include a number of Poaceae, which occur in a latitudinal range from 24°N to 47°N in North America; the sparseness of endemics at high latitudes is probably due to the more recent exposure or re-exposure of glacial outcrops as plant habitats.

Turner and Powell [161] state that "comparison is needed between diploid ancestral types versus tetraploid-derived counterparts of gypsous and non-gypsiferous plants. Preliminary data indicate (from chromosome counts of 125 species of 55 genera) that, within a given taxonomic group, diploids are more common among gypsiferous taxa than among their non-gypsiferous counterparts. Gypsophylic elements may thus be more relict in their specialized habitats.

Some closely related taxa also grow on saline soils; some of the endemics may reflect a physiological jump from saline to gypsum soils. Even so, the morphological differentiation of these contrasting species suggests that the availability of such habitats has been sufficiently long to trap ancestral relics, or else their occupancy was early enough, so as to provide sufficient time for morphological differentiation via micro-adaptational mechanisms.

Probably relevant to the further study of the origin and evolution of endemism in South Asia is a paper on edaphic endemism by Raven [117], referring to an earlier essay of 1962 by Harlan Lewis—a model for speciation in a marginal populations under conditions of extreme selective pressure and concomitant severe reduction in population size. Following the trend towards increased aridity operative in south-west North America since at least the mid-Oligocene, numerous pockets of species usually found in moist regions have been cut off. Such isolation has been accentuated since the Pleistocene and Recent times by increase in altitude.

> "In effect, the region constitutes a gigantic 'tension zone' between temperate and tropical biota . . . It therefore appears reasonable that this process as outlined by Lewis has played a large role in the proliferation of the narrow endemics so characteristic of the region. As much as 29 per cent of the vascular plants of California are endemic—an amazing proportion for a continental region . . . I suggest that there may be a direct connection between the edaphic endemism so prevalent in dry regions and catastrophic selection . . . Many workers suggest strongly that in plants, the occupation of unusual soil types almost inevitably involves the formation of genetically distinct races . . . Marginal populations apt to be growing on substrates, unusual for the species as a whole would be the very ones most likely to undergo catastrophic selection, and as a result be fixed at adaptive modes different from those of the main body of the species."

2.6 CELL AND NUCLEAR EVOLUTION

Takhtajan [152] was convinced that the monocotyledons were derived

from dicotyledons, having acquired their characters while they adapted strongly during a phase of evolution to aquatic or at least marshy habitats. The main groups have gained in homogeneity and most orders are placed in a logical sequence . . . in Commelinacae. The orders are placed in a series from a more Lilialean to an advanced Poalean sequence, with the Restionales just above Poales.

Dahlgren believes that the closest group to the grasses is *Joinvillea* (Joinvilleaceae), followed by *Flagellaria* (Flagellariaceae). Other closely related groups are the Restionaceae (in which Ecdeiocolea and Anarthriaceae should be included) and Centrolepidaceae. "It would not surprise me either, if grasses and palms shared a common ancestry far back in the middle Cretaceous" (Dahlgren, personal communication, 1986 [38]).

According to Stebbins [144] the earliest grasses "were herbs with stems having few to many nodes, relatively simple racemose or paniculate inflorescences, and spikelets with numerous florets, the bracts or glumes being undifferentiated like those of primitive bamboos". He disagreed with Prat [107] and Bews [8] that the bamboos were ancestral to the other tribes, believing with Arber [3] that their tree-like habit was a secondary specialization [183]. Stebbins considers the grasses existed in the middle Cretaceous in tropical or subtropical climates, and that from that extinct group, a series of lines adapted to different habitats evolved. "Three of these lines had unusual success. The first two developed primarily in the tropics. These were Panicoideae and the Chloridoid-Eragrostoid line", probably from closely related ancestors, which evolved by retaining primitive leaf epidermis, caryopsis, embryo and seedling and specialization in leaf anatomy. The third dominant line, the Festucoideae, are the principal grasses of temperate climates. Their large chromosomes are probably a later specialization [4]. Stebbins notes that while some temperate orders have small chromosomes, the Commelinaceae, Liliales, Leguminosae and Polemoniales also have large chromosomes. "The relationship between chromosome size, cellular metabolism and growth is a subject which certainly deserves attention, and about which new discoveries of considerable evolutionary significance might be made" [144].

Many of the less successful line, the bamboos, dominant only in the moist forests of the tropics, retained primitive reproductive structures [186] but others are highly specialized. The Arundineae retained the primitive basic chromosomal number of $x = 6$ or 12 [183]. The Oryzeae have retained the primitive six stamens per floret, while the Stipeae, "probably an offshoot of the danthonoid-arundinoid complex, became highly specialized in fruit structure" [144].

In his review of chromosome evolution in the monocotyledons, Sharma [130] refers to Avdulov's recognition of three types of grass karyotypes: $x = 9$ and 12, both with small chromosomes, and $x = 7$ with large chromosomes. Sharma notes that while polyploids have an advantage in new environments, they are less adapted than diploids to their centres of origin.

> "In the millets, and including *Triticum* and *Avena*, the chromosome numbers are 14, 28 and 42. In the Paniceae they are $2n = 72$, whereas in the andropogons the somatic number ranges from 20 to 180, all in

multiples of ten. The genus *Bromus*, which is one of the primitive taxa of the festucoid complex, has $2n = 14$ in some of the species."

"On the basis of observations so far made, multiples of $x = 5, 6, 7$ and 10 chromosomes are most prevalent in the gresses. . . . . In general chromosomal details of grasses present several interesting features. A high degree of polyploidy is common to many genera. Both structural and numerical alterations of chromosomes have been frequently observed. Interspecific hybridization is not uncommon. Large numbers of cytotypes, especially aneuploid complexes, have been recorded in several genera such as *Cenchrus, Dicanthium, Bothriochloa* and *Poa* [130, 184].

2.7 POLYPLOIDY

Polyploids may be the result of some form of abnormal meiosis in diploids or other lower ploidy levels, when reproduction took place under the influence of some form of stress, especially aridity. Conversely, polyploidy confers certain benefits upon plants which enable them better to meet extreme ecological conditions. Stebbins [147] has noted correlations between polyploidy and occupation of pioneer, disturbed habitats.

It has already been proposed (see Sec. 2.2) that environmental stress has been a major factor in the evolution of the rich gramineous flora of western monsoon Asia—250 genera and 1,250 species [12]. Preliminary indications are that, the forms of abnormal meiosis that may be found in, for example, the species of the plains of western monsoon Asia are associated with relatively slight, sometimes difficult-to-identify but nevertheless significant changes in the chromosome complement of the progeny. But the many plains genera and species which show polyploid series may be presumed to have had an environment-induced origin at some period of history*.

Mehra [87] in a collection of tetraploid accessions of the *Dichanthium annulatum* complex from almost its entire range of distribution; observed some meiotic irregularities ranging from slight, medium or marked, and of the type commonly associated with polyploidy and hybridization between species. But Mehra and Singh [88] explain the chromosomal behaviour noted in the four ecotypes of the *D. annulatum* complex, which they recognize (tropical, Mediterranean, Senegalese and South African) and their hybrids entirely on the basis of cytogenetical data. The forces which attract chromosomes together, which initiate pairing, and which govern behaviour at meiosis are all believed to be genotypically controlled. There is no reference on the microscale of the possible action of environmental factors operating through the nutrition and metabolism of the plant up to the tapetum and so to the

* This section had remained incomplete at the time of the author's death. He had wished to discuss findings from the following papers here:

Anton, A.M., A.E. Cocucci. 1984. The grass megagametophyte and its possible phylogenetic implications. *Pl. Syst. Evol.* **146:** 117–21.

Crawford, D.J. 1985. Electrophoretic data and plant speciation. *Systematic Botany*, **10:** 405–416.

Ghorai, A., A. Sharma. 1981. Chromosome studies in some Festuceae. *J. Indian Bot. Soc.* **60:** 148–53.

meiocytes which it commands, nor on the macroscale to the geographical cytology of the ecotypes growing in their so contrasting environments.

Where the ecoclimatic border is along an altitudinal zone or contour, a new level of ploidy may be met. For example, Janaki Ammal [71] reviewed the effect of the Himalayan uplift (introducing an association of operative factors other than the aridity/rising temperatures complex stressed here for lower altitudes) in changing the chromosome complex of genera of families other than the Gramineae, which were members of the flora of Laurasia before the Himalaya reached their present height. All polyploids must originally have arisen from diploids, except in the rare cases of reversion by parthenogenesis. High polyploidy is noted in regions near the glaciers of the eastern Himalaya, where the range bends over the plateau of Assam, a region also favouring hybridization at the place of meeting of Indian, Chinese and Malesian floras.

The evolutionary history of the grass flora of the Great Plains of North America is interpreted by Stebbins on the basis of the probable origin and development of eight different polyploid complexes that include common species of this flora. At least some of the modern grasses that inhabit the Great Plains have entered the area from different directions: the mountains in the western United States, Mexico, south-western United States, Asia. The evolution of the grasses in the North American plains repeats a story that is now familiar . . . Since evolution results primarily from population/environment interactions, the greater are the environmental changes, the more drastic will be the alterations of the fauna and flora of a region [146].

Sharma [133] has examined the overall percentage of polyploidy in Himalayan grasses in general and also reviewed whether this polyploidy is related to changes in latitude and climate as between the eastern, central and western parts of the Himalaya. Stebbins [144] estimated that nearly 70 per cent of grass species are polyploids, or more if one includes genera with chromosome numbers which are also derived by ploidy during their evolutionary history. Polyploidy must not necessarily be taken to be only a recent phenomenon; Ehrendorfer and collaborators [45, 46] refer to palaeoploidy, often the result of profound shifts and reductions in habitat and distribution area. Stebbins [147] considers it a common phenomenon in vascular plants.

Of 697 taxa of Himalayan grasses, 63.4% were polyploids, the incidence decreasing with increasing latitudes, together with change from subtropical (in the east) to sub-temperate (central) and temperate (west, with the Kashmir Hills) climates [133]. Sharma therefore does not agree with the proposal by Love and Love [84] that there is a tendency for an increase in polyploidy in angiosperms with increase in latitude.

2.8 APOMIXIS

Apomixis is a secondary manifestation of what was initially some type of physiological imbalance between bisexual ancestors and their environment, causing failed meiosis in the ovules combined with failure of pollen development in the anthers. Is it correct to talk about genetic control of a derived condition—apomixis—and not about the genetic basis for a physiological capacity or incapacity to react in a certain way to various forms of stress, in

various directions associated with abnormal meiosis, only one of which is apomixis? It would be desirable for physiologists to attempt to dilute or eliminate manifestations of apomixis by appropriately designed and timed nutritional experiments on bisexual plants which show a regular or spasmodic tendency to produce apomicts in their progeny. Special attention should be given to example of seasonal and population differences [78].

Bhanwra and Choda [93, 80 references] have extended the earlier review of apomicts in the angiosperms [101], in which 91 grasses belonging to 30 genera were listed as showing apomixis. The recent paper enumerates apomictic taxa which have been reported since Nygren's review, including many South Asian grasses in the tribes: Andropogoneae, Maydeae and Paniceae in the subfamily Panicoideae: Arundineae, Aveneae, Bromeae, Eragrosteae and Phalarideae in the subfamily Festucoideae. The types of apomixis recognized are: apospory, adventitious embryony, diplospory and parthenogenesis, with the unreduced embryo sac being four-nucleate or eight-nucleate. For example, it appears that about 94 per cent of the apomictic species in the Andropogoneae are aposporous and form four-nucleate embryo sacs; these embryo sacs generally possess one egg, two synergids and one polar nucleus, or rarely one egg, one synergid and two polar nuclei. All are pseudogamous, with triploid endosperm in *Bothriochloa odorata, Capillipedium huegelli, Dichanthium annulatum* and *Eremopogon foveolatus.* Polyembryony, due to multiple embryo sacs, has been reported in several species.

Reddy [118] considers apomixis in Pooideae to be primitive, with structures formed as a result of this mechanism being much closer to sexual reproduction. "On the other hand, the mechanism of apomixis has progressively evolved in Panicoideae, reaching the zenith in Andropogoneae with Paniceae as an intermediary."

Bhanwra and Choda [9] believe that spontaneous hybridization between species and genera is essential for the origin of apomixis within a plant group. Most of the apomicts of Reddy in Festucoideae are diplosporous and form eight-nucleate embryo sacs, whereas all the apomicts in the Panicoideae (except *Tripsacum dactyloids*) are aposporous. The apospory occurs in *Agropyron scaber, Chloris* species and *Hierochloe* species, and four-nucleate embryo sacs are found in some species of the tribes Chlorideae and Eragrosteae [16].

Jeanmonod [75] regards apomixis as an *echappatoir a travers deux modes,* vegetative reproduction and agamospermy. The importance of the latter phenomenon is proved by the large number of agamospermous plants of which many are polyploid. The correlation which exists between agamospermy and hybridization is demonstrated by the presence among agamosperms of a high level of heterozygotes, of hybrid swarms, or uneven polyploids (thus sterile) and of methods of fertilization similar to those of interspecific hybrids [54, 103, 142]. Agamospermous plants do not usually cross, but there are some fertile agamospermous hybrids which are able both to produce fertilized seed and seed resulting from agamospermy in varying proportions. This occurs, for example, with *Hieracium aurantiacum* [140].

According to Grant [54], agamospermy has the advantage of producing

copies of a heterozygous genotype with good adaptability, but which would be lost in normal sexual reproduction. The combined processes of natural hybridization and agamospermy lead to the formation of micro species. The resulting structure is an agamic complex, in which the derived agamospermous hybrids are superimposed on the original sexual species. The genus *Poa* is mentioned among the classical examples. Agamospermous forms are generally polyploid and more widely distributed than the original diploid species.

Shishkinskaya and Larina [137] detected abnormality in the development and structure of the female gametophyte in the polyploid species, *Festuca gigantea, F. valesiaca* and *Koeleria sahuletorum*; most of these corresponded to embryological characteristics associated with apomixis.

Bothriochloa odorata (tetroploid, $4n = 40$), and *Paspalum distichum* (hexaploid, $6n = 60$), both developed aposporous embryo sacs with a pseudogamous mode of reproduction. The megaspore mother cell degenerates as such, or soon after the formation of tetrad. As many as one to five aposporous embryo sacs arise from the cells of the nucleus. The embryo separates, developing from the diploid egg cell. It fails to reach maturity in the ovules where there is no endosperm formation. In *Bothriochloa odorata* the endosperm is triploid and is formed as a result of fusion between diploid pollen nucleus and a male gamete. In *Paspalum distichum* it is pentaploid, as both polar nuclei take part in fertilization [26].

If apomixis is to be regarded as response of ancestral gass types to different forms and intensities of environmental stress, a distinction may be drawn between different types of stress in the environments in which the Panicoideae and Festucoideae originally grew and where they occur today.

Much of the current work on apomixis is related to its use in plant breeding. This is discussed fully in the case studies which follow, with special reference to *Pennisetum* and *Panicum*.

Apomixis "has provided a dynamic reproductive mechanism for genome preservation, polyploid build-up through fertilization of unreduced eggs (the apomict functioning as the male parent in crosses with sexual plants) and survival of derivatives of wide crosses. Brief contact between cross-compatible sexual and apomictic plants has resulted in interspecific transfer of apomixis in these genera. Hybridization of sexual *Cenchrus ciliaris* and *C. setigerus* resulted in a vast array of sexual and apomictic types, some of which might easily be classified as new species if discovered in natural habitat. Obligate apomixis is genetically controlled in *C. ciliaris* and *C. ciliaris* × *C. setigerus*. Two pairs of genus and epistasis are involved."

The primary interest at the moment is in the possibility of controlling and manipulating this "unique reproductive process" in modern grass breeding [6].

2.9 PHOTOSYNTHETIC PATHWAYS*

The only known fossil grass leaves showing both external micromorphological and internal anatomical features on the same specimens have been

* The author would like to thank Dr. P.W. Hattersley for valuable assistance in this section.

described from the Late Miocene of Nebraska [159]. The internal anatomy of the fossils is typical of grasses using the C_3 photosynthetic pathway, and the combination of characters suggests taxonomic relationships with members of the Arundinoideae (see also [157, 158]).

The origin and evolution of C_4 photosynthesis is an intriguing problem in biology. It is found in at least 18 families of angiosperms, both monocotyledons and dicotyledons, with the majority occurring in the grasses. A fossil leaf fragment collected from the Ogalla formation of north-west Kansas exhibits features found in taxa of the modern grass subfamily Chloridiodeae, including Kranz leaf anatomy. The leaf fragments extend the fossil record of plants that show both anatomical and external morphological features indicating C_4 photosynthesis back to the Miocene [185].

The designation of the Poaceae of South Asia according to their photosynthetic pathways into C_3 and C_4 types is an aspect of plant physiology, which should be considered in the present context. There are, moreover, three biochemical subtypes of C_4 photosynthesis, which differ, in decarboxylation enzyme and site of decarboxylation within the PCR cells known in the Poaceae [60]. Two species of *Panicum* [39] differed in C_4 acid decarboxylation type and in developmental origin of the bundle sheaths in major veins of their leaf blades. A fuller review of the biochemical, physiological and cytological features in C_3 and C_4 grasses has been planned.

Hattersley [61] analyzed the distribution of C_3 and C_4 grasses in Australia in relation to climate. "All but four of 833 native and 292 naturalized Australian grasses have been assigned as having the C_3 or C_4 photosynthetic pathway. . . . In general, C_4 grass species, like C_3 species *increase* in number with increasing rainfall, in their preferred temperature regime. C_4 species are most numerous where the summer is hot and wet; C_3 species where the spring is cool and wet. C_4 species numbers decline with increasing temperature and/or decreasing spring rainfall" (see also [64, 65]).

Hattersley (in press) concludes that there is little that can be said about the evolution of C_4 photosynthesis in plants that is not speculative (see also [10, 15, 112, 141]). Monson *et al.* [99] and Holaday and Chollet [69] have reviewed C_3–C_4 intermediacy.

In their study of the classification of the Poaceae into subfamilies and supertribes, Watson *et al.* [168] review the taxonomic distribution of C_4 photosynthesis—"not a single taxonomic feature but a complex phenomenon or syndrome; this must have arisen separately in several unrelated families, including the Poaceae. The switch from C_3 to C_4 (and/or the reverse?) must have occurred on several occasions. This classification presents one entirely C_4 supertribe (Andropogonanae), one entirely C_4 subfamily (Chloridoideae) (but see Ellis, [47]), two mixed C_3–C_4 subfamilies (Panicoideae and Arundinoideae—rather distant from one another), and one mixed supertribe (Panicanae). The C_4 syndrome appears to be homologous across the grasses. Switching from C_3 to C_4 involves drastic leaf-structural and spatial reorganizations involving mesophyll and mestome sheaths, vascular bundles and suberized lamellae [62, 168]; also precise changes in the localization and kinetics of the Rubisco enzyme and extensive redeployment of pyruvate

orthophosphate dikinase. "The probability of arriving at the complete C_4 syndrome and the same variants more than once (let alone several times), by independent evolution via selection of chance mutations must be very small; and it seems improbable that the syndrome should have a different genetic basis from group to group" [168]. Two alternatives to completely independent evolution are suggested: can "one presuppose that the capability for switching to C_4 arose early in the history of the grasses, to be somehow preserved in an inoperative (or not noticeably operative) state, or could there have been clandestine transfer of genetic information?"

3. Some Case Studies

Agrostis

Bor [12] lists 35 species and varieties in South Asia, and Cope [37] lists eight in Pakistan. This is a genus of 150 to 200 species, mainly in temperate and cold regions, especially in the northern hemisphere—generally at higher altitudes in the Himalayan and associated ranges to the west, and in the Nilgiris, annual and perennial types exist.

Rajbhandari [113] examined specimens of nine species from high altitudes in Nepal, mostly in extreme cold/arid environments on alpine and subalpine grasslands. These species are all annuals. Most botanists agree that annuals arose from perennials (E.J.H. Corner, personal communication, 1976), particularly in areas exposed to different forms of physiological stress. One may therefore ask (in relation to Himalayan orogeny) where are or were the nearest perennial forms of *Agrostis, Calamagrostis* or *Deyeuxia,* from which these annual forms evolved, somewhere at a very early geological age, when the Himalayas were lower in altitude? If any Gramineae were present at lower altitudes when the climate was tropical/subtropical before uplift began, or even during the early progressive stages of uplift, they are most unlikely to have been species of these genera. Did *Agrostis* and its close relatives of temperate adaptation come from the west (the Irano-Turanian region) or from the north, along the ancient routes of plant migration from the north of Laurasia, which were followed by other temperate genera and species of the high Himalaya, before these routes were cut by the desiccation resulting from the rise of the Himalaya?

Cenchrus

Bor [12] recognizes seven annual and perennial species in South Asia; Cope [37] as many as five in Pakistan. "*Cenchrus* is distinguished from *Pennisetum* by the transformation of the involucral bristles into a spiny cup. The tendency is barely recognizable in *C. ciliaris,* whose membership of this genus is justified by its intergradation with *C. pennisetiformis*" [37].

Sexual and aposporous apomictic embryo sac development in three *C. ciliaris* genotypes was evaluated *in situ* and *in vitro* at the U.S. Regional Pasture Research Laboratory, Pa 16802 [57]. The *in vitro* culture technique provides a method for studying embryo sac development and physiologic regulation of apomixis. Few investigations into the physiological basis of apomixis have been reported [57, 136, 2·08].

Zeven and de Wet [176] state that *C. biflorus* ($2n = 30, 34, 36$) and *C. ciliaris* ($2n$ = mainly 36) and probably also *C. setigerus* ($2n = 34, 36, 37$) are of African origin; if so, do we have to visualize migration from east Africa to the west and north of South Asia, either by man-borne trade or by land along the Hadramaut and Makran coast? If this is proved, would one not expect to find a graduation from fully sexual to obligate and facultative apomictic types along the migration route, from the African centre of origin into the biological vacuum of the Eocene?

Coix (Maydeae)

Bor [12] gives specific rank to *C. aquatica*: although difficult to separate from *C. gigantea* in the herbarium, they have different habits and different chromosome numbers (*C. aquatica* $2n = 10$; *C. gigantea* $2n = 20$). *C. lacryma jobi* is a primitive cereal cultivated in many parts of the tropics. *C. puellarum* is common in south-east Asia (187).

A.B. Sapre and associates at the Botany Department, Marathwada University, Aurangabad, observed chromosomal range in interspecific Indian hybrids [125], meiotic non-disjunction in *C. gigantea* [124], spontaneous emergence of parents in hybrids [126], a spontaneous autotriploid in *C. aquatica* [121], and a group of studies on aneuploids and higher polysomics (nullisomy to pentasomy and hexasomy [122–124]).

Although *Coix* is generally regarded as a primitive genus, there is no reference to cytological criteria for primitiveness. Sapre and Deshpande [126] have, however, noted that one or two chromosomes from *gigantea* species (G) showed behaviour like B-chromosomes when incorporated in the *C. aquatica* genomes (AA) through spontaneous hybridization.

Cymbopogon

Cytological observations are being made at the C.S.I.R. Regional Research Laboratory, Jammu Tawi, India, by V. Verma, S.N. Sobti and associates on the genus *Cymbopogon* (Andropogoneae), a polymorphous group of aromatic grasses—140 species in the tropics of the eastern hemisphere, 20 in India in regions with abundant rainfall, from sea level to 2,000 m elevation. There are nine varieties, cytotypes and chemotypes belonging to six species. Of these, five were diploids ($2n$=20), two were tetraploids ($2n$=40), and two were hexaploids ($2n = 60$). Chromosomes are classified into three groups on the basis of size. Variation in the karyotypes of different species is mainly due to variation in number of chromosomes belonging to these groups.

Karyotypic evolution appears to be taking place through structural differences and repatterning of chromosomes along with general reduction in total chromatin length. On the basis of hybridization studies and karymorphological differences between the two varieties of *martinii* (*motia* and *sofia*), it is suggested that the two varieties be raised to specific status [163].

The base number of chromosomes is found to be $x = 10$, with the possibility of accessory or B-chromosomes. A basic number of $x = 5$ has been suggested for the Andropogoneae, but, being unstable, this became duplicated in the evolution of the tribe [89, 92, 93].

Six species and their varieties (five diploids, one tetraploid and two hexaploids) show morphological diversity, possibly due to gene exchange. Both the diploids and the hexaploids showed fairly normal meiosis with 10 and 30 bivalents respectively. However, the tetraploid species (*C. martinii*, $2n = 40$), formed multivalent configurations, suggesting a segmental polyploid nature [164].

The presence of a comparatively symmetrical karyotype in certain species suggests that they are closely related to the ancestral stock. The karyotypes of comparatively modern species, having varying degrees of symmetry, might have originated from the above ancestral forms by structural changes in the chromosomes and their repatterning, including increase and decrease in size [46, 143, 145].

Dactylis

Dactylis *glomerata* is an important grass of the mountains to the north of South Asia at altitudes with a temperate climate (1,800 to 3,600 m). Cope [37] has not attempted to resolve the intraspecific taxa in Pakistan and Kashmir, but distinguishes the following intergrading subspecies:

subsp. *glomerata* ($2n = 28$) occurs throughout the range of the species;
subsp. *bispicata*, also tetraploid, occurring mainly in the southern and western part of the range of the species, but reaching Afghanistan and possibly Pakistan;
subsp. *himalayensis*, diploid, the most common variant in Pakistan, restricted to the western Himalayas.

In all diploid forms of the species and their F_1 hybrids, meiosis was regular [151]. In all the tetraploid studies, chromosome behaviour resembled that in typical *D. glomerata*, with the formation of a varying number of quadrivalents and bivalents, followed by regular chromosome segregation. The diploid species which the authors discuss constitute only about 5% of the genus; the tetraploids make up the remaining 95%; the latter may have evolved as the result of hybridization, but the high proportion may also be an expression of cytological response to stress at some past stage of migration and evolution.

The use of isozymes to indicate intraspecific groupings has been applied to *Dactylis glomerata* [85]. The results show that most diploid or tetraploid subspecies can be classified in one of the two main geographical groups (Eurasian or Mediterranean), differentiation between them probably having occurred long ago, probably in the Miocene. Diploid and tetraploid subspecies within each climatic group show some similarity, but the genetic diversity is always higher in tetraploids than in diploids. The analogies could be due to phylogenetic relations and/or adaptive convergence. The additional alleles found in tetraploids would be due to the inertia of the tetraploid genetic structure, which slows the rate of segregation. The genetic structure of the Eurasian *himalayensis* and *aschesoniana* subspecies was found to be very close; it is likely that they have a common origin [85].

R. Lumaret (CEPE/CNRS, B.P. 5051, 34033, Montpellier, France) reports in a personal communication (August, 1986) that she has received material from the district of Xianning, north-west Guizhou, collected at 2,200 m. This

material is diploid; no tetraploids have yet been found. The Chinese type is similar in panicle characters to *Dactylis glomerata* ssp. *himalayensis*, supporting the hypothesis that these two types originated from a common ancestor, but there are differences in the leaves. There is no divergence between the types in the chloroplast DNA structure from DNA restriction fragment patterns. Enzyme polymorphism has been studied at six different loci, which code for different types of enzymes. Surprisingly, at the level of each locus, fixation of one allele, sometimes two, was found. This has never before been reported for the genus *Dactylis*. There may have been a dramatic differentiation for this entity, which would indicate a long and significant isolation.

Until sufficient evidence has accumulated, it may be proposed that the types of *D. glomerata* found in the western Himalayas entered this new mountain habitat from the Irano-Turanian region, while those described by Bor [12] and maintained in the herbaria of the Forest Research Institute and the Botanical Survey of India in Dehra Dun reached the Himalaya from the north; they may have been components of the vegetation of the Xizang plateau before the final uplift of the Himalaya along its southern rim and the introduction of increased aridity in the plateau north of the range. In this connection, reference should also be made to the possible significance of the Chitral/Gilgit gap as a dividing line between western and eastern Himalaya, or to the differential rate of speciation through polyploidy in the north-eastern and north-western Himalaya—low stress versus high stress [71].

Dactyloctenium

M.L. Sharma and associates in the Department of Botany, Panjab University, Chandigarh, India, have studied several species and cytotypes of the genus—five species in India, annual and perennial, Eragrosteae. Seed sterility in *D. sindicum* may be due to failure of the pollen tube to reach the embryo sac in most of the ovules, and failure of normal megasporogenesis and megagametogenesis in a low percentage of ovules.

It is most difficult to separate the annuals, *D. aegyptium* and *D. aristatum* using Bor's [12] criteria, as many intermediate forms also occur. On the basis of cytotaxonomic studies on 81 populations from the plains of Chandigarh and eastern Punjab, it is concluded that the *D. aegyptium* complex in the Punjab plains comprises taxa belonging to *D. aegyptium* (L.) P. Beauv. and *D. aristatum* Link., and populations which represent new varieties of *D. aristatum*. A taxonomic key is provided [132, 134]. Leaf epidermal characters have little diagnostic value either at the specific or subspecific level [135].

Cope [37] recognizes three species in Pakistan, *sindicum*, *aegyptium* and *aristatum*, but notes that the boundaries between species are often ill-defined. "Further collecting and some experimental work are required before this genus can be fully understood."

Panicum

The most primitive representative of the Paniceae, *Pseudolasiacus*, occurs in Madagascar, while most of the genera considered to belong to the same tribe, Microcalamineae, are also African [20, 21]. Bor [12] recognized 32

species as authentic in the South Asian types of the genus *Panicum*, but noted 375 synonyms proposed for specific rank by other taxonomists. Does this indicate degree of speciation within the African genus in response to new environments [173]? Looked at from the South Asian subcontinent, one tends to regard the mesophytic population in India, e.g. *Panicum maximum*, as of early origin on the Plate, and the species of more xerophytic adaptation like *P. antidotale* and *P. turgidum* as more recent arrivals in western monsoon Asia from east and north-east Africa (see also *Cenchrus* in these Case Studies).

Scanning electron microscopy, used to study the anthoecia of *Panicum elegans* from Late Cenozoic deposits in Kansas, revealing that the germination lid has been present in some members of the Gramineae since at least the Late Miocene-Early Pliocene [155].

Current interest in this genus relates to the genetical use of apomixis in breeding procedures. Savidan [128] reported that 500 accessions of *Panicum maximum* collected by Combes and Pernes [36] comprised, when grown in Cote d'Ivoire, 22 sexual diploids ($2n = 16$) among a population predominantly of tetraploids ($2n = 32$) reproducing by apomixis. Hybridization between sexual and apomictic plants gives rise to sexual as well as apomictic hybrids.

The character of a four-nucleate embryo sac structure was used in genetical analysis to differentiate apomitic from sexual hybrids. Sexual biotypes contain only eight-nucleate embryo sacs. All the data from ten different types of progenies fit with a single gene model for the inheritance of apomixis, in which the sexual diploids would have the genotype aa and the apomictic tetraploids the genotype Aaaa; apomixis is dominant. Apomixis in *P. maximum* is often facultative, but with a low percentage of residual sexuality, a rate that remains low following hybridization.

Using the same criteria for sexuality and apomixis (Savidan's simplified embryo sac analyses) in trials with *P. maximum* at the National Grassland Research Institute, Nishinasuno, Japan, Nakajima and Mochizuki [99] recognized how degree of sexuality could be used in a breeding programme: (1) crossing a sexual plant with an apomict to expand genetic variations through genetic recombination, and (2) selecting promising hybrid plants in which heterosis has become fixed by apomixis.

Sinha and others [138, 139] examined two cytotypes with $2n = 18$ or 32. Savidan (personal communication, September, 1986) considers $2n = 18$ "extremely doubtful; all examined cases corresponded to taxonomical mistakes". Meiosis was irregular, characterized by the presence of univalents at metaphase I and laggards at anaphase I. Pollen sterility was high; a number of pollen mother cells also showed desynapsis. The mode of reproduction may be correlated with the meiotic behaviour of chromosomes and the fertility of the gametes.

Scientists in the Department of Agronomy and Botany in the University of Georgia (Athens, GA 30602) have studied the photosynthesis, leaf anatomy, morphology and cytogenetics of hybrids between C_3 and C_3/C_4 species within the *laxa* group of *Panicum*.

"There were no differences found in morphology or physiology between the amphiploids and the F_1 hybrids from which they were produced. In the segregating progeny, CO_2 compensation concentration and photorespiration values typical of C_3, but not of C_3/C_4 plants were recovered. Progeny were found from both crosses which possessed O_2 inhibition of apparent photosynthesis typical of the parents, and in the case of the *P. milioides* × *P. laxum* cross, leaf anatomy and overall plant morphology typical of the parents was observed in some progeny. The progeny were found to possess recombinations of various traits associated with reduced photorespiration, so that no correlation existed among O_2 inhibition of apparent photosynthesis, CO_2 compensation concentration, and leaf anatomical traits. One plant was especially noteworthy in possessing leaf anatomy typical of C_3/C_4 plants, but with CO_2 exchange characteristics of C_3 plants" [14].

*Paspalum**

Of this genus, of about 250 species distributed throughout the tropics but mainly in the New World, Cope [37] recognizes three species in Pakistan: *P. dilatatum* (introduced from South America), *P. paspalodes* (closely resembling the more tropical *P. vaginatum*) and *P. scrobiculatum* (a highly polymorphic species which may be a swarm of apomicts) [32]. Accepting 14 species of the genus in South Asia, Bor [12] states that *P. dilatatum* was introduced from South America into India, and has now become established in many hill stations.

Chromosome numbers of most *Paspalum* species are multiples of 10; apomixis is prevalent within the genus and has been an important factor in the evolution of many species [18]. Common *P. dilatatum* is an apomictic, 50-chromosome natural hybrid with three genomes that pair as 20 bivalents and 10 univalents at meiosis. In an effort to circumvent apomixis, Burson [17] undertook a phylogenetic investigation to identify its progenitors, and eventually to resynthesize the species. The genome formulae II, JJ and IIJJ have been assigned to *P. intermedium*, *P. jurgensii* and the yellow-anthered biotype of *P. dilatatum* respectively, of which the first two species or others closely related may be the ancestors. It was proposed that common *P. dilatatum* (IIJJX) arose from a cross between a sexual tetraploid and an apomictic hexaploid, which may have been *P. durifolium* or another hexaploid species in the section Quadrifaria [17].

A series of papers by Argentinian (Instituto de Botanica del Nordeste, 3400, Corrientes) and American cytologists will be of interest to workers in South Asia (most recent reference [108]). More than 400 species are recognized. Aspects covered include (1) chromosome behaviour, embryo sac development and fertility of *P. modestum* ($2n = 20$), *P. boscianum* ($2n = 40$) and *P. conspersum* ($2n = 60$), wild species which reproduce sexually and may have forage potential; (2) cytogenetic studies on *P. laxum* ($2n = 60$), *P. proliferum* ($2n = 40$) and 20 and 40 chromosome accessions of *P. cromyorrhizon*—meiotic behaviour and embryo sac development suggest that *P. laxum* is a sexual

* The author is indebted to Dr. Byron Burson for valuable comments and amplification of this section.

allohexaploid and *P. proliferum* an apomictic autotetraploid; $x = 5$ may be the primitive number from which species with $x = 6$ and $x = 10$ were derived; tetraploid *P. cromyorrhizon* is a facultative apomict, whereas the diploid is primarily sexual, but appears to have some potential for apomictic reproduction; (3) cytogenetic relations among *P. notatum* var. *saurae*, *P. pumilum*, *P. indecorum* and *P. vaginatum*—the actual relation of *P. indecorum* to the other species is at present unclear; (4) cytology of intra- and interspecific hybrids between diploid and tetraploid cytotypes of *P. notatum* and *P. cromyorrhizon* indicate they are genomically related, but the sterility of their diploid × diploid hybrid confirmed as separate species.

Most species of the Quadrifaria group are distributed in tropical and subtropical South America [109]. *P. quadrifarium*, a member of the group is distributed mainly between 30 and 39°S. lat. in Argentina, Uruguay and South Brazil. It probably also originated in warm regions, but migration to temperate or less humid regions was accomplished by means of polyploidy and apomixis.

Pennisetum

Cytological investigations on some of the Indian millets, including *P. typhoides* and *P. orientale*, were carried out by Sharma and De [131], who discussed the implications of meiotic irregularities in *P. typhoides*. Recent work on the cytogenetics of this crop of semi-arid environments in Africa and Asia, *P. typhoides*,* has been fully reviewed by Hauhar (U.S.D.A. Agricultural Research Service, Albany, GA 94710). Chromosomal hybridization and genetic studies have shown that the wild or semi-wild relatives of pearl millet are not sufficiently isolated from it to deserve specific rank. All annual Penicillarias (a section of the genus *Pennisetum*) have therefore been merged with *P. typhoides*, in which three subspecides are recognized.

There are several semi-wild annual diploid races in the section Penicillaria with which the cultivated pearl millet is interfertile, and essentially forms a single, composite reproductive unit. There is no reference to the possible perennial ancestor of these annual types of *Pennisetum*, which would presumably have arisen in the drought-affected fringes of areas in which perennials were and perhaps still are dominant in the natural vegetation [170].

Based on the more frequent occurrence of B chromosomes in primitive varieties than in selected, commercial varieties, it has been suggested that their occurrence might be used as an indicator of a crop's centre of origin. On that basis, the Sudan [105] and Nigeria [19] have been proposed as the centre of origin of pearl millet, but Jauher [73] does not agree, preferring the Sahel—a good borderline for genotypical response to fluctuating environments (see Sec. 2.3).

All Penicillaria fall into the $x = 7$ group. The only perennial species, *P. purpureum* is tetraploid, and possesses a genome partially homologous to pearl millet (Dujardin, personal communication, September 1986); all other

* M. Dujardin, University of Geogia College of Agriculture, Tifton, Georgia 31793, disagrees with Jauhar's use of the species name *typhoides*, and considers *P. americanum*, as used by taxonomists such as Clayton, to be correct.

taxa are annual and diploid with $2n = 2x = 14$ chromosomes. In his section on abnormal meiosis, Jauhar [72–74], discusses desynapsis and its genetic basis, the effects of nutrients and ploidy on desynapsis, the effect of the desynaptic gene on B chromosomes and the experimental induction of desynapsis. Other sections of the review deal with haploidy, polyploidy, aneuploids, structural changes in chromosomes and floral biology (protogyny and anthesis). The development of the megagametophyte ahead of the pollen meiocytes is an advantage in outbreeding of pearl millet [114].

The somewhat anomalous occurrence of certain grass species throughout the high Himalayas, Xizang and north into innermost Asia has to be considered in any attempt to use botanical evidence for the Gondwanian origin of parts of the Asian landscape and vegetation. One may regard the genus *Pennisetum* as of Gondwanian (African) origin. Yet Bor [12] refers to *P. lanatum* in the western Himalayas and in Xizang above 1,500 m; also to *P. flaccidum* (= *P. incomptum*) in the Himalayas and Xizing at high altitudes. The staff of the Kashmir substation of the Indian Grassland and Fodder Research Institute reports on a tall form of *P. flaccidum* in the Kashmir Valley and a stunted form at higher altitudes in Ladakh. Cope [37] refers to the distribution of the species as "Pakistan (Sind, Baluchistan, Punjab, Northwest Frontier Province, Gilgit and Kashmir at elevations up to 2,700 to 3,000 m); Himalaya westward to Afghanistan, Tibet and southern China". The Gramineae volume of the Flora of the U.S.S.R. [119] notes the occurrence of *P. flaccidum* in Central Asia, Pamir, Altai, with general distribution in Iran, India (Himalaya), China (Junggar Pendi/Kashgar), Mongolia. Keng Yili [77] gives the distribution of *P. flaccidum* as north, north-west, north-east and south-west China, in addition to Central Asia. Wang Chiwu [167] reports the occurrence of the species in a herbaceous cover on exposed slopes in the valley plains of southern Chahar and Suiyan in association with species of *Artemisia* (approximately 40°N latitude; altitude 800 to 1,000 m). Modern surveyors have noted that *P. flaccidum* forms a steppe on the right (southern) bank of the Yarlung Jiang (Brahmaputra) in Xizang, on the northern slopes of the Himalaya at 4,000 m adjacent to a steppe of the Leurasian *Stipa* [25].

The apomictic East African grass, *P. squamulatum* ($2n = 54$) was crossed to tetraploid pearl millet ($2n = 28$) to study potential for germplasm exchange [42]. Fertile apomictic interspecific hybrids appear to be a useful tool for the transfer of genes for apomixis from the wild species to pearl millet. The production of partially male-fertile apomictic back-crosses (*P. americanum* $2n = 4x = 28 \times$ *P. squamulatum* $2n = 6x = 54$) should make it possible to continue gene transfer from the wild species to the crop in succeeding generations [43].

Other research at the University of Georgia College of Agriculture relates to apomictic interspecific hybrids between pearl millet and *P. orientale*, meiotic and reproductive behaviour of facultative apomictic back-cross offspring derived from *P. americanum* × *P. orientale* interspecific hybrids, microsporogenesis, reproductive behaviour and fertility in five *Pennisetum* species, cytogenetics of double-cross hybrids between *P. americanum-P. purpureum*

amphiploids and *P. americanum* × *P. squamulatum* interspecific hybrids, pseudogamous parthogenesis and fertilization of a pearl millet × *P. orientale* apomictic derivative, and cytology and reproductive behaviour of pearl millet × napier grass hexaploids × *P. squamulatum* trispecific hybrids [44].

Poa

A cosmopolitan genus of 200 species, mainly in temperate regions or altitudes in the Northern Hemisphere, "a very difficult genus to name, partly on account of the wide range of variation displayed, partly because many of the species are closely related" [37]. The relative members of 32 recognized 3 species and 49 synonyms in Pakistan, and the comparable figures for India (species or varieties, 52, synonyms 84) may perhaps be taken as a measure of active speciation [12]. Partial or complete breakdown of meiosis has been noted in *P. alpina, P. bulbosa, P. nemoralis* and *P. pratensis* in the western Himalaya [94].

Kellogg [76], in her biosystematic study of the *Poa secunda* complex, notes that about a quarter of the species occur in the Pamirs and the Himalayas [59]. Some species are fully sexual, others are partial or obligate apomicts; both inbreeding and fully outbreeding and dioecious species also occur [30]. Taxa are recognized primarily on the bases of size of parts and the presence or absence of trichomes and scabrosities in various parts of the plant. The core of any taxonomic revision must therefore involve a detailed analysis of variation in these characters.

Even in the offspring of self-pollinated plants, Kellogg found much morphological variation.

> This variation "could be entirely phenotypic, entirely, genotypic or some combination of the two. If all the offspring of a single plant were apomictically produced, they would be genetically identical, assuming no autosegregation or mitotic crossing-over. Although all the plants were greenhouse-grown, the experiment was not controlled for small differences in environment. At the other extreme, the variation could be all genetic. If all the seeds were sexually produced, then they could all be genetically different. . . . No matter how the variation is explained, however, it is still high enough to suggest that the characters are of minimal taxonomic value. . . .
>
> "In general, my work suggests that much of the presently accepted taxonomy of the genus *Poa* is suspect. The amount of population-level variability in the *P. secunda* complex is not unique in the genus. The genus contains several widespread polymorphic taxa, including *P. pratensis, P. alpina, P. arctica* and *P. glauca*, in which one to many species are commonly recognized. All include numerous entities that have been given specific status at some time in the past, and all are circumboreal, apomictic, and with aneuploid chromosome numbers suggesting some ancestral hybridizations.
>
> "At our current level of knowledge, we can make no claims about evolution within *Poa secunda*. Because there are no characters that can serve as evolutionary markers, we cannot evaluate the various processes

that might have generated the pattern. Hypotheses of fusion of disparate lineages by hybridization, although appealing, are merely plausible suggestions, not subject to test. The roles of polyploidy and apomixis cannot be evaluated. The pattern of variation, in other words, does not illuminate the historical pattern of microevolutionary process" [76].

Rottboellia

"The delimitation of the genera within the group of taxa represented by *Rottboellia* and its closest relatives. . . . has always posed a considerable problem" [162]. Stapf raised Hackel's system of five subgenera to generic rank, creating several new ones, and since that time, the number of genera has steadily increased. Despite the work of many taxonomists, difficult questions remain. A clarification of the genera which occur in Malesia has been made, on the basis of morphological characters and some chromosome counts. . . . "but clearly the last word has not been said. A really stable situation will only be reached when far more data are available, e.g. from anatomy, cytology and, if possible, hybridization experiments," [162].

Christopher [27, 28], University of Kerala, Trivandrum, India, has studied polyploidy in the *R. exaltata* complex in South India [12]. The genus has about 30 annual and perennial species in tropical and subtropical regions of the world (Cope [37] says only four species), and *R. exaltata* is the only species in India and Pakistan. Taxonomists recognize a short and a tall, robust varieties (short $2n = 20$; tall, $2n = 40$). Both showed normal meiosis, with regular bivalent formation, anaphase separation and formation of normal tetrads. The tetraploid taxon is a natural allopolyploid; the short diploid form may be one of its parents. In south India, the species is a compilospecies, the diploid form being very restricted in distribution, the tetraploid widespread.

Sporobolus

The record of $n = 6$ in the pollen mother cells of *S. maderaspatanus* (collected in south India, [29] confirms that for *S. molleri* ($2n = 12$) from East Africa—a comparatively rare basic number in the Gramineae [153]. Meiosis was regular and six bivalents were observed at metaphase I; 90.5% of the pollen was fertile and seed set regular.

Cope [37] notes that *Sporobolus* is a genus of about 150 species of annuals and perennials, mainly in the tropics and subtropics, with some species extending to warm temperate regions; 10 in Pakistan. "A large genus not divisible into well-defined sections, although clusters of closely allied species are readily apparent. Within the clusters, and to a lesser extent between them, the boundaries between species are seldom clear-cut, and the occurrence of intermediates appears to be the rule rather than the exception."

Stipa

Morphological studies of fossil grass anthoecia from Miocene-Pliocene strata in Kansas are cited by Thomasson [156] as evidence for the common ancestry of North American species of *Stipa*, section *Hesperostipa*, and of *Piptochaetium* in *Bothriochloa*. Both species have been distinct from species of *Nassella*, *Oryzopsis* and other *Stipa* since at least the Miocene or Pliocene.

The taxonomic revision of the genus presented by Freitag ([50] University of Kassel, D3500, Federal Republic of Germany) for the area between the east Mediterranean (south-west Asia) and the Himalaya up to Nepal will be of value to Indian botanists, and to Chinese specialists concerned with the genus on the north face of the Himalaya and areas to the north in Qinghai/Xizang. The northern boundary of Freitag's area agrees with that of the *Flora Iranica** for the central part of the area; to the east, it follows the frontiers of Pakistan and India, and in the north-west the main range of the Great Caucasus. The southern boundary is the most natural, since it is here that the genus gradually disappears in the subtropical deserts, semi-deserts, woodlands and forests of western monsoon Asia. The author is familiar with most of the species from his stay in Afghanistan and field visits to the Himalaya, Iran and Transcaucasia.

Concepts differ widely regarding the delimitation of the genus—very narrow by most Soviet authors, wide by western ones [12, 37]. Freitag's revision results in 42 accepted species for the region, includes about 50% of all Euroasiatic species, and covers almost all infrageneric taxa present in Eurasia. The frequently separated genera, *Achnatherum*, *Lasiagrostis* and *Ptilagrostis* are now included in the genus *Stipa*. The significance of the growth form is related to the mode of branching, the ontogenetic development of the shoots and the life span of the individual, with the following types (among others) being recognized: non-rosulate perennials, rosulate perennials and rosulate annuals.

The genus *Stipa* is adapted to temperate, dry climates from lowlands to alpine belt, and exhibits greater diversity in semi-arid and arid climates. About 40% of the species are considered rare, either very restricted endemics, perhaps collected only once and therefore of doubtful status, or they have come from adjacent regions—a few outposts located in marginal areas.

Primitive characters including chromosome numbers of $2n = 24$ are more or less equally distributed in sections *Lasiagrostis* and *Aristella*. The alpine offshoot *Ptilagrostis* has $2n = 22$. The Mediterranean sect. *Macrochloa* is an isolated group of obscure origin with more primitive characters. Freitag's Table 5 : 378 gives presumed evolutionary trends in some characters of the genus, primitive and advanced. Primitive chromosome numbers are 22, 24, 28; advanced numbers are 36, 38, 44.

Cytological data are few, but it is evident that polyploidy is much involved in the evolution of the genus and its sections. However, there is only a limited correlation between chromosome numbers and the height of morphological organization. "Much more cytotaxonomic work is needed to detect the evolution of species and the relationship of the sections within the genus *Stipa*" [50].

*Rechinger, K.H. (ed.) 1970. *Flora Iranica* no. 70: Gramineae. Graz.

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9

Metals as Clastogens—Some Aspects of Study

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1. Introduction

Toxic effects of certain metals have been known from the beginning of civilization. However, interest was intensified in their action on cellular systems after the realization of the increasing exposure of living organisms to metals in the environment as a result of growing industrialisation [71, 74]. Industrial activities have disturbed geological caches of heavy metals, such as, Cd, Hg, Tl and Pb, to name a few, releasing high amounts into the atmosphere from the underground resources within a short period. The pollutants are deposited on soil, water and biota from the aerial load. Additional amounts are deposited directly from different sources, such as manures or refuse. Processes like precipitation, gravitational settling and absorption are responsible for the transportation of such metals to great distances from the site of introduction in both aquatic and terrestrial environments.

Other human activities have also enhanced appreciably the levels of the more common metals in the environment. Certain instances are:

— mercury cathodes in the chlor-alkali plants produce 20–47 ppb and reagent grade sodium hydroxide plants give 53–1290 ppb of mercury [78].
— the amount of mercury released from coal-burning is 80 times that from geothermal sources [2].
— man-made emissions, in fact, account for 25 to 30% of the world-wide burden of mercury during the past century.
— mercury concentrations in sediments from freshwater lakes and estuaries are two to five times higher than those before the inception of human culture [85].

Similarly, Cd occurs in proportions between 0.1 to 0.2 ppm in the earth's crust. It is however widely distributed as sulphide and oxide ore deposits and is present in the ocean in small quantities [63]. Specific populations, like metal workers, receive a much higher level of exposure and the risks of cytotoxic effects are on the increase [100].

The toxic effects of metals on living organisms are expressed at different levels—pathological, histological, cellular, sub-cellular or molecular [72A, 72B, 89]. The expression is modified by a number of variables, including the state of health of the organism—plant, animal microbe or man [23].

2. Mode of Action

The action of metals on cellular systems usually involves several stages, before final expression.

At the level of membranes, metals may:

— affect the permeability of the cell membrane or act as antimetabolites.
— affect the mitochondrial membrane and disturb energy metabolism.
— decrease the stability of the lysosomal membrane, leading to a disruption of cell functions by the release of acid hydrolases [79].

At the molecular level, the effects may involve:

— interaction with proteins, resulting in denaturation, precipitation, allosteric effects and/or enzyme inhibition.
— binding to nucleic acids, leading to irreversible conformational changes. Interaction with DNA may culminate in mutations or even carcinogenesis.

These effects may be manifested, at certain stages, as alterations in chromosome configuration, chromosome behaviour and disturbance in spindle formation and cell division. In some cases, these changes may become heritable.

According to a schematic model occasionally used to describe the behaviour of Fe^{3+} and other transition elements in different cells, and in extracellular fluids of living organisms, metallic ions are suggested to exist in four different forms, namely,

— as free ions,
— as low molecular weight complexes,
— as reversible macromolecular complexes and
— as irreversible macromolecular complexes ([54], see Fig. 1)

With elements like Al^{3+}, low molecular weight complexes with organic acids, amino-acids, nucleotides, phosphates or carbohydrates, are often chelates and may be very stable. Simultaneous binding may occur for more than one chelate—forming ligand, and these may be the intermediate forms where the ligands are exchanged. The reversible macromolecular complexes are formed with a large number of polyanionic macromolecules, like proteins, polynucleotides, glycosaminoglycans and so on. Since with some metals, like Al^{3+} which has a high affinity for such compounds, these complexes are much less active than the small ones, they may be responsible for the lowering of the metabolism of the metal. On the other hand, certain complexes formed between metals and macromolecular structures may be so stable as to be almost irreversible and are only destroyed with the loss of the structure binding the ion. Complex formation with stable structures results in the accumulation of the ion in the tissue concerned. Usually these complexes are found in the final destination for the particular metal in the cell.

The charge density also influences the existence of metals in anionic and cationic forms. For example, the very high charge density of Al^{3+}, permits it to bind to almost any oxygen or nitrogen atom in the biological environment.

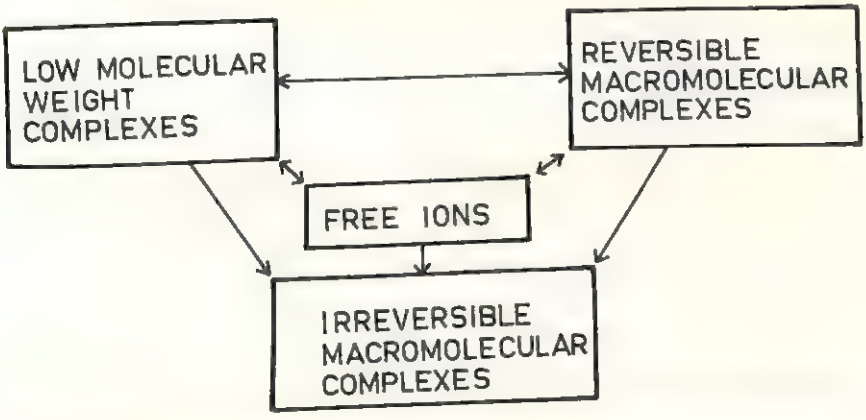

Fig. 1: Schematic model of behaviour of metal ions at intra- and extracellular levels (Ganrot [23]).

An even higher charge density, as in Si and B, increases the tendency of the ion to bind oxygen to such a level that it ceases to exist as a cation and can function only as a complex anion.

The phenomenon of chemical cooperativity has also been recorded in certain metals, e.g. Cr^{3+}, where the binding of additional cations to collagen increases the stability of complexes already formed, being irreversible at a particular threshold value [40]. Even below the threshold value of the cation/polyanion ratio, the binding can become irreversible by the clustering of the cations, to exceed the threshold values, so that more and more polyanions and cations are bound to the growing complex. The process is similar to crystallization in a supersaturated solution after the crystallisation nucleus is formed. Under *in vivo* conditions, it may be most suitable for the cell nuclei, where polyanionic DNA is present in a very high concentration, or for the collagen-rich connective tissue [23].

The most important action of metals, from the point of view of genetical studies is on the genetic material, particularly DNA. A large number of potentially reactive sites for metals are present in nucleic acids, thus explaining the fact that different workers have often assigned a given ionization or complexation step to different sites on the same species. This uncertainty in the assignment of the sites of proton and metal ion attachment to these species is often compounded by variable experimental conditions, like ionic strength, presence of other competing ions as well as different types of base pairing and base stacking in the DNA.

Earlier workers had divided the different ligands—purine and pyrimidine bases, nucleosides, nucleotides, and polynucleotides—into two groups according to their general affinity for metal ions, namely, those which contained phosphate moieties and those which did not. Similarly, metal ions could be classified according to their affinity for ribo-nucleotides into three types: those which interact almost only with the base portion, those which interact with both base and phosphate and those which interact almost exclusively with the phosphate part. However, it was later observed that the binding could better be expressed in terms of degree of a particular metal with both phosphate and base. The degrees vary between metal ions (1A, 110).

In general, alkali metals were found to bind exclusively to the phosphate moieties of ribonucleotides and DNA. Most alkaline earth metals interact only with the phosphate moiety in the ribonucleotides, polyribonucleotides, DNA and RNA. The transition metal ions are present in, and extremely tightly bound to, the naturally occurring RNA material. They may be responsible for holding the RNA molecules in specific conformations. In these bivalent metal ions, an increasing degree of affinity has been observed for the base relative to the phosphate site in the sequence Co^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} (1A, 74).

3. Expression of Cytotoxic Effects

The interest in metal cytotoxicity has been further enhanced following the

information gatheced on the genotoxicity and possible carcinogenicity of a number of metals [101]. For example, particles of metallic Ni, Ni_2S, and NiO are regarded as respiratory carcinogens in nickel refineries [83] and arsenic has been reported to cause epidermoid carcinomas of the skin and lungs [59A]. Other metals, like Se, have shown both carcinogenic and anticarcinogenic effects. Metals causing genotoxicity also often induce carcinogenesis finally [22]. There is a positive correlation between the degree of mutagenecity and the incidence of malignancy [45], as has been recorded for other chemical toxicants as well (see [35]).

3.1. ACTION ON THE GENETIC MATERIAL PARTICULARLY DNA

The interaction of metals with DNA, as mentioned earlier, comprises a major fraction of the research on understanding metal carcinogenesis (see [6, 31]). The genotoxic events have been studied extensively particularly those which involve,

— inhibition of DNA semiconservative analysis;
— production of DNA damage, such as DNA adducts, base loss, strand breaks, interstrand cross-links and DNA-protein cross-links;
— effects on chromosome structure, as shown by sister chromatid exchanges, chromosomal aberrations and the formation of micronuclei.

A large number of techniques have been evolved to identify and quantify the alterations induced at the level of chromosomes (see [3, 18, 34, 73, 74, 76, 77, 82], for details). Different parameters require to be screened and monitored, in order to assess the risk of damage following exposure [100].

Such studies on genotoxicity of metal ions are complicated by their high affinity for all classes of cellular compounds, including cell organelles (see [81], for review). The competition between toxic and essential metal ions for binding sites on proteins and other macromolecules critical for cellular function, is also a major aggravating factor. Chemical reactivity, as shown by their physicochemical softness is directly proportional to the LD_{50} of most metal ions in animal systems [60, 92]. The more toxic ions, like Cd^{2+} and Hg^{2+}, tend to form bonds of a more covalent nature, because of their easily deformable outer electron shells; on the other hand, the bonds formed by essential metals like Ca^{2+} and Mg^{2+} are predominantly ionic in character, since these are hard ions. The formation constants of hard ions with model ligands may be lower than those of the soft ions by two to five times [36]. The relatively higher toxicity of the metal ions having a higher level of chemical softness may be associated with their capacity to form more stable complexes with cellular components then the essential ions, thus disturbing and preventing the normal metal-mediated cellular functions.

This close relationship between general toxicity and the reactivity of metals is not so clear with respect to genotoxicity and carcinogenicity, because of numerous other factors involved. The effects of metals, as far as can be seen while screening for DNA lesions, or alterations in DNA repair synthesis, indicate the possibility of their ultimate involvement in carcinogenesis [6]. In estimating the carcinogenic potential of individual metals, certain other factors come into play. Some examples are given below:

i) The early transition metals, like Cr(VI) and Mn(VII) exist as anionic oxygen or hydroxy complexes, chromate CrO_4^{-2} and MnO_4^-, at low concentrations in neutral aqueous solution [37]. Their entry and subsequent intracellular reactions will differ from the divalent metal cationic complexes formed by Ni^{2+}, Cd^{2+}, or Hg^{2+}(56A, 56B).

ii) The specificity of the interaction of a metal with the critical target like DNA will determine its potential as carcinogen. In this connection, differences between reactivity with the bases as against the phosphate groups in DNA will affect the transforming potential.

iii) The concentrations involved in gross toxicity and genotoxicity are not related. The latter may occur at cellular concentrations well below that causing cytotoxicity. Alternatively, a highly toxic dose may even be responsible for a transforming event.

In vivo studies have shown that developmental stages are more susceptible to metal-ion treatment than adult ones, possibly due to the blockage of critical macromolecular synthesis [7]. Flow cytometry has indicated that a number of inorganic metal compounds block cells throughout the S phase. These compounds include both $NiCl_2$ and crystalline NiS and $CdCl_2$, $HgCl_2$ and $PbSO_4$ [29]. Since any effect on the synthesis of either RNA or protein could finally affect DNA synthesis, the blocking of the S-phase by a number of metal ions does not indicate a specificity of action or similarity in the mode of action. For example, treatment of isolated rat liver cells with $CdCl_2$ inhibited nucleolar *r*RNA synthesis to a greater extent than *hn* RNA synthesis [66]. In most cases, protein synthesis inhibitors also alter the synthesis of nucleic acids, making it difficult to assess the most critical site of action for the effects on DNA and RNA [67B]. In a relatively few cases, the structural characteristics of the molecule permit specific reactivity with a particular macromolecule, without affecting any others, as for example, the specific affinity of diamino- dichloro- platinum for DNA.

Damages to DNA, induced by several metals, have been identified through alkaline sucrose gradient centrifugation and alkaline elution techniques [42]. The latter is more sensitive to lower levels of DNA breakage. Most metals induce single strand breaks in DNA, though the effectivity varies with the form used. For example, trivalent Cr, rather than the hexavalent form, was the DNA- damaging agent in treatments of isolated human embryonic lung fibroblast nuclei with $CrCl_3$ (Cr^{3+}) and $K_2Cr_2O_4$(Cr^{6+}). The higher potency of the trivalent oxidation state in reacting with DNA explains the metabolic conversion of Cr^{6+} from the readily absorbed $K_2Cr_2O_4$ to Cr^{3+} by a possible microsomal metabolic oxidation [25].

3.2. ACTION ON CHROMOSOMES AND CELL DIVISION

Changes in the chromosomes may involve alterations in the number or structure or both. The metals causing chromosome break or translocation are termed *clastogens* while alterations in number are principally due to the *spindle poisons*. The end effects often overlap.

The cytogenetic criteria for identifying clastogenic activity involve the

measurement of chromosome breakages and sister chromatid exchanges (SCE), singly or in combination. For quantitation of the effects, chromatid aberrations, induced following breaks at G_2 phase, are more suitable than chromosomal aberrations recorded at the first metaphase after treatment. Thus a short-term incubation, to cover only part of the S and the G_2 phases, both *in vivo* and *in vitro,* is more desirable than long-term incubation.

The sister chromatid exchanges (SCE) are usually induced at doses below those leading to increased chromosomal aberrations [44, 94]. This parameter, under both *in vivo* and *in vitro* conditions, in combination with cytogenetic assays, is able to detect mutagenicity at very low levels [64].

Micronucleus test, initially developed for bone marrow cells *in vivo,* can identify chromosome breaks induced in the preceding division. It is suitable for routine screening due to its relative simplicity and rapidity (see [53]).

Chromosomes from male germ cells in animals are useful only for the individual, but not for routine screening. In some cases, interphase chromatin can be monitored for premature chromosome condensation (see [33]).

Though the hazards posed by metallic pollutants are different in different organisms, yet the induction of genetic toxicity, that is, the ability to damage DNA is a common phenomenon in most of them. Numerous techniques have been devised to assess the extent of genetic toxicity. The most common are short-term bioassays, employing microbial test systems, with or without microbial enzyme inactivation, for point mutations. More elaborate are the methods utilizing higher organisms for measuring clastogenicity and alterations of divisional frequency *in vivo. In vitro* protocols range from studies of the effects on plant and animal tissues in culture or on isolated cells.

The clastogenic activities of metals show certain general trends. The mitotic index usually decreases with increase in concentration of the chemical used and the duration of the treatment. The spindle formation is often disturbed, leading to metaphase arrest, stathmokinesis, formation of diplochromatids, lagging and ultimately polyploidy in some cases. In this process, both the affinities of metals for reactive groups in cytoplasmic proteins, leading to the formation of metallothioneins, and alterations in the osmotic pressure may be involved.

The damage induced by changes in chromosomes may differ. The alterations may be *minor,* showing gap, lesion, fragmentation and/or translocation. They could be *moderate* or *gross,* with extensive damage to chromatin matter, non-specific in nature, culminating in ultimate lethality to the cell. These alterations necessarily overlap and depend largely on the dosage and the period of exposure, within the threshold values. The initial action may be specifically on the DNA or protein or non-specific in nature.

Such changes, whether in mitotic index or chromosome or spindle behaviour, indicate that most metals, when administered to higher organisms, are clastogenic at certain concentrations and durations of treatment. The inherent toxicity depends on the capacity to disturb cellular metabolism by reacting with cell organelles, macro-molecules and metabolites. The initiation of the effects and the degree of manifestation are associated with a number of

factors, including the type of the test system used; the rate and mode of administration; transport and distribution in the tissues; the vehicle used; the physical form of the metal in the target tissue; the rates of detoxification, excretion and interaction, both with foreign and endogenous substances. Additional factors are tissue specificity [58] and the physiological condition of the organism. The final expression of the effect depends also on the mode of action of the metal itself with the different bio-organic molecules (see [74]).

A general assessment of the data available from mammalian systems shows that the inherent toxicity of the metals, *within* each vertical group of the periodic table, is directly proportional to increase in atomic weight, electropositivity and solubility of the metallic cations in water and lipids in relation to conditions within the tissue, except Li and Ba. The pattern of inherent toxicity, on the basis of horizontal periods in the periodic chart, increases with successive periods. Within a given period, the inherent toxicity of the metal cation decreases progressively from groups I to IV, followed by a gradual increase in that of the metal anion from groups V to VII. In group VIII, both ions have a similar level of toxicity. The increased solubility of the alkali salts of the oxy-acids of metals of groups V to VII enhances this inherent toxicity (see [74] for details).

The information, as yet available, indicates that the effects of different metals and their compounds on chromosome and cell division relate well with the relative toxicity of the chemicals, as assessed by the physiological and histological criteria (see [13, 14, 15, 19, 20, 56, 57, 68], for details).

As expected, in plant systems, the solubility of the chemical in water is of much greater importance than in mammalian test systems. In general, in plants the cations and the degree of dissociation of inorganic metallic salts quantitatively affect the frequency of the aberrations produced.

From their effects on cell division in plants, metals can be classified into three categories, according to the descending order of activity:

i) *Very strong effects*: Tl, Cd, Cu, Hg, Cr, Co, Ni, Pt, Pd, Be, Ag and Au.

ii) *Very active metals*: Zn, Al, Ca, Mn, Fe, Se, Rb, Sr, Sb, Th and U.

iii) *Relatively inactive metals*: B, Na, K, Mg, V, As, Mo, Ba, Pb and Bi.

The relative clastogenicity of some metals, as shown following exposure of *Allium cepa* roots to different doses, were: As > Se > Mo; Co > Ni; Cu > Mg > Mn; > Pb > Sr > Ce: Hg > Cd [76, 80, 86, 87]. This classification was upheld from tests of clastogenic effects in mammalian systems as well [26, 28]. In *Allium cepa*, salts of groups IV and VII were significantly mitostatic as compared to control, while those of II and VIII were slightly so. In *Rattus norvegicus*, on the other hand, inorganic salts of group V decreased the mitotic index significantly as compared to the control. Within a group, in general, the mitostatic effect increased with increasing atomic weight.

In plant systems, the frequency of chromosomal abnormalities induced was significantly higher than the control with inorganic metal salts of groups IV and VII, but not with III. In animals, only the metals of group V induced a significant increase [27].

Newer techniques are being evolved to compare the genotoxicity and clastogenicity of metals. Some of the more promising ones include:

— The displacement of acridine orange (AO) from DNA through the interaction of metals with DNA, as measured by fluorescence polarisation [69]; less than 0.5 mM concentrations of Pb, Mn, Co, Zn, Cd, Ni, Fe, Cu, and cis-Pt displace 50% of the AO. The alkali metals, on the other hand, require a concentration of more than 50 mM to displace an equivalent amount of AO from calf thymus DNA.

— Progressive computerization and standardization of data obtained by different screening techniques [88].

Techniques involving different parameters to study cytotoxicity often tally quite closely [67A, 74].

4. Interaction Between Metals

The cytotoxicity of a metal is greatly influenced by the presence of other metals. The net result may be synergistic, antagonistic or simply additive. The phenomenon of antagonism and synergism between metal ions had been known earlier at the level of mammalian tissues, e.g. the well-known antagonism between Se and Hg; Zn and Cd [24, 90]. In mammalian diet, Zn and Cu protect against heavy toxic doses of Cd. Vanadium toxicity is intensified by high dietary Zn but decreased by Mn. Interactions of Cd with Cu and Zn and Fe in mammalian tissues can cause symptoms associated with Zn, Cu and Fe deficiencies and can affect Cd toxicity, depending upon the relative availability of the different metal ions. In plant systems, such information is lacking. The basic mechanisms for antagonism and synergism are inhibition or promotion of either the formation of toxic metabolites or the process of detoxification. Interaction is the overall mechanism involved. This aspect has been utilized to reduce metal toxicity in some cases, e.g. excretion of Se from mammalian tissues was decreased by Zn but increased by Te, As, Hg, Tl, Co and Cd (see [89]).

Works carried out in the authors, laboratory have indicated significant changes in the clastogenicity of a metal when it is used in combination with another metal of the same group. These results again depend on the concentration used, the mode and sequence of administration and the test system. The clastogenic activities of Hg and Pb can be counteracted in mammalian systems by Se but there is no effect in plants (see [5, 13, 14, 20, 57B, 72A, B]).

5. Scope for Future Work

5.1 IN AGE RELATED PROCESSES

The ubiquitous involvement of metals in biological functions has generated considerable research on their role in the ageing processes.

In this aspect, further information on the cumulative effects of non-essential forms of metals on cellular ageing would be needed. Heterochromatization has often been suggested to be an essential process in cellular ageing (fig. 2), but no reliable explanation has yet been proposed [12, 84, 96]. Alumi-

nium for example has been shown to bind to DNA and chromatin to form stable irreversible complexes and its accumulation has been suggested by some workers to be an important factor in the ageing process [21]. This idea is based on the ability of Al^{3+} to cross-link macromolecules, particularly collagen. The turnover rate of collagen is very slow and believed to be caused by progressive increase in cross-linking between the molecules, leading to a decreased ability to be degraded by collagenase [41, 70].

DNA cross-linking has been suggested to be a part of the ageing phenomenon, as also number and rate of replication of DNA strands, as seen in tissue cultures from different organs (see [97, 98] for review). The well-known action of most metals in leading, directly or indirectly, to DNA cross-linking, on gradual accumulation, adds to the importance of this study.

Certain diseases, known to exhibit symptoms of senility as compared to the normal ageing process, are of importance in understanding the factors underlying senescence. For example, senile dementia of Alzheimer's type (SDAT) is regarded to be the most common cause of brain failure in the elderly [1, 38, 65].

In some cases, a genetic predisposition has been noted [91], suggesting the presence of two genes. In patients, the amount of heterochromatin of neurons and glial cells was reported to be greater than that in normal subjects [11]. Also the neuronal RNA content was decreased from 25 to 50% [52, 49, 50], with a concurrent reduction in nucleolar size [17, 51]. Fluorescent-histochemical studies have indicated a doubling of Al^{3+} concentration in heterochromatin from neurons of SDAT patients [9, 10, 26]. These evidences support the hypothesis that SDAT may be due to a slow accumulation of Al^{3+} in the brain.

In the initiation of Endemic amytrophic lateral sclerosis (ALS) and Parkinsonism-Dementia, environmental factors have been regarded as more important than hereditary ones [93] and metals have been cited as likely agents, with other cultural ones. Alpha particle induced X-ray fluorescence studies have recorded enhanced amounts of Al, Si, P, Ca, Ti, V, Mn and Fe in the brain and spinal cords from patients with endemic ALS [95]. Infection and metal exposure have been mentioned as possible causes for the classic ALS as well, Hg, Pb and Mn being suspected [43].

In ALS-affected neurons, both decrease of RNA [4, 16, 30, 48], and alteration of DNA to inhibit transcription [4] have been recorded. The morphological effects are decrease in nucleolar size and increase in heterochromatin.

Chronic Mn poisoning produces symptoms similar to Idiopathic Parkinsonism and even dementia. Mn^{3+}/Mn^{2+} could give rise to cytotoxic substances by catalyzing the auto-oxidation of dopamine [99].

5.2 IN STUDY OF DISEASES OF MULTIFACTORIAL CAUSES

Metal cations often induce hypersensitivity, in which a complex of the ion (hapten) and a larger molecule (carrier), usually a protein, are involved. There is a comparatively low specificity for the cation, but a high specificity for the carrier [8].

For this reason, metal allergies have sometimes been compared with

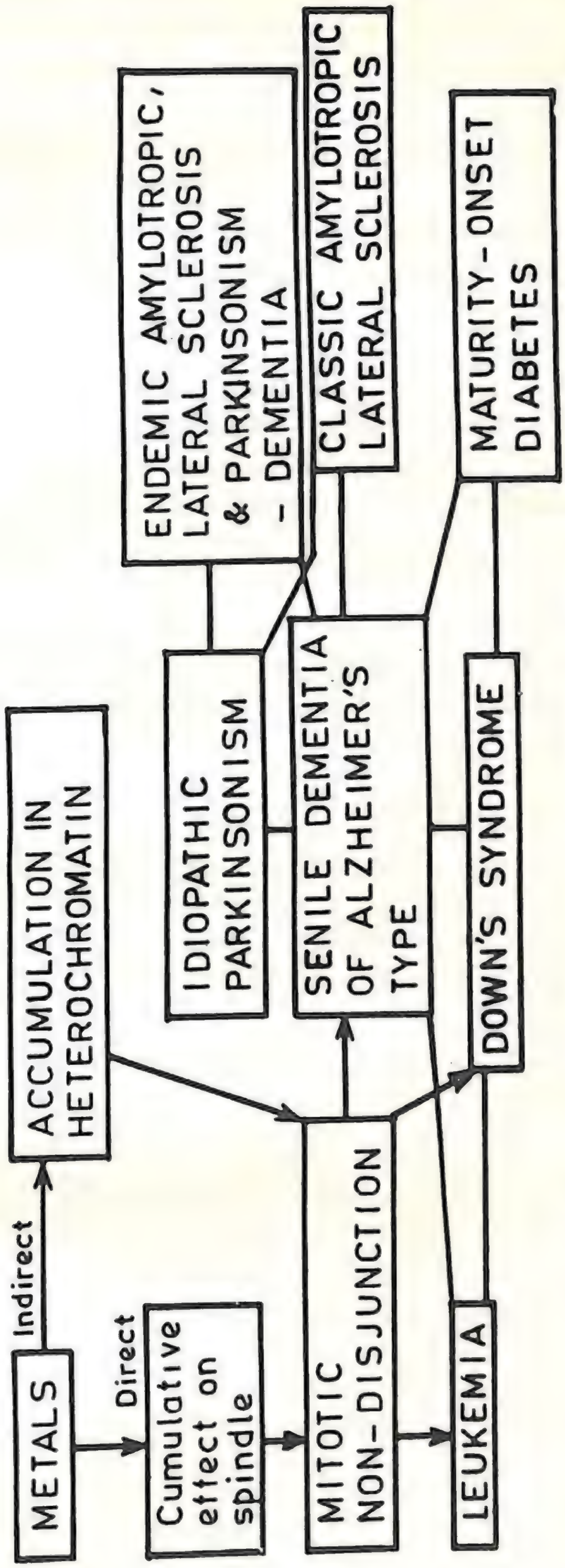

Fig. 2: Epidemiological connections between some age-related diseases.

autoimmune conditions. Hypersensitivity induced by metal cations is usually of the delayed, cell-mediated (type IV) type, that clinically causes eczema or granuloma. However, even within the same group, Cr^{3+}, Be^{2+} and Zr^{4+} are strong allergens but Al^{3+} is not.

Two other examples of known or suspected association of specific metals with diseases are:

i) accumulation of Ga^{3+} in active sarcoidosis foci,
ii) increase of Ca^{2+} and Ga^{3+} in muscle cells from patients with muscular dystrophies.

5.3. IN ASSESSMENT OF EXTERNAL MODIFYING FACTORS

The effects of external factors, like the presence of other toxic chemicals, including metals in the atmosphere, the nutritional level, the prevalence of other infections and addictions often complicate monitoring for metal cytotoxicity in exposed human populations. Even under *in vitro* conditions nitrolotriacetic acid trisodium salt (NTA)—a component of household laundry detergents—increased the SCE frequency significantly, induced following treatment of mammalian cell cultures by insoluble salts of Cd, Ni, Hg and Pb. However, the frequency of SCE induced by soluble salts of the same metals was not affected [47, 55].

5.4. IN STUDY OF GENETIC ADAPTATION

Natural populations, subjected to chronic environmental pollution, may develop increased resistance to toxicants, either

— through physiological acclimation for one set of individuals, or
— through the action of natural selection on genetically based individual variation in resistance.

Enhanced resistance has been recorded in most, but not all, aquatic populations subjected to heavy metal pollution ([102], for review) but the genetic basis cannot be confirmed in all cases. Evolution of such resistance is less conclusive in metazoans than in micro-organisms. In general, resistance differences in bacteria, algae and fungi appeared to have a genetic basis, whereas physiological acclimation could not be distinguished from genetic adaptation in metazoans. The absence of resistance has led to the reduction of taxonomic diversity and in the number of species in metal-polluted environments, as for example of terrestrial plants [105].

The physiological mechanism for metal tolerance may involve:
(i) A reduced accumulation of metals by the organism, including an increased transformation in resistant bacteria of the highly toxic methyl mercury to the less toxic ionic Hg^{2+} and then to the least toxic and volatile Hg [104]. (ii) Release of extracellular metabolites which act as chelaters to reduce the effective metal concentration available [105]. (iii) Sequestration of the metal in a less toxic form or site. It may be carried out by binding the metals to the inducible cytosolic metallothioneins [106]. Another metal complexing compound was isolated from plants [107]. Increased levels of metallothio-

neins in metal-resistant mammalian cell lines and metallothionein-like proteins in cadmium-adapted oligochaetes [108] and bacteria [109] indicate the means through which the toxicity of metals can be counteracted by different organisms.

Further information is needed on the metal resistance of higher organisms and the minimal thresholds of exposure, particularly susceptibility of individual groups.

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The editors have jointly written *Chromosome Techniques–Theory and Practice* (Butterworths, 3rd edition, 1980), and edited a two-volume series on *Chromosome Evolution in Eukaryotic Groups* (CRC Press). They edit jointly *The Nucleus*–an International Journal of Cytology (est. 1958).

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